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FINAL REPORT

Study Title

Evaluation of a Test Article in the *Salmonella typhimurium/Escherichia coli* Plate Incorporation Mutation Assay in the Presence and Absence of Induced Rat Liver S-9

Test Article

3-Nitro-1,2,4-Triazol-5-one (NTO)

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Laboratory Project I.D.

SITEK Study No.: 0985-2140

Study Initiation Date

August 26, 2008

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October 17, 2008

Sponsor

US Army Center for Health Promotion and Preventive Medicine
Aberdeen Proving Ground, MD 21010

Sponsor's Study Coordinator

Gunda Reddy, Ph.D., DABT

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14. ABSTRACT

3-Nitro-1,2,4-triazol-5-one (NTO, 99.6% pure) was tested for mutagenic potentials with Salmonella typhimurium strains, TA 98, TA 100, TA 1535, TA 1537 and Escherichia coli strain WP2 uvrA by plate incorporation method according to OECD TG 471 in compliance with Good Laboratory Practice. NTO was tested at concentrations of 5, 10, 50, 100 and 250 ?g/plate for Salmonella strains and 100, 250, 500, 750 and 1000 ?g/plate for Escherichia coli without activation. The dose levels were 100, 500, 1000, 2500 and 5000 ?g/plate for both Salmonella and Escherichia coli with activation. The results showed that NTO was not mutagenic in Salmonella typhimurium and Escherichia coli both with and without activation. The negative result was also verified by a confirmatory mutation assay both with and without activation.

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STUDY DIRECTOR'S COMPLIANCE STATEMENT

Study No.: 0985-2140
Sponsor's Test Article I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

The protocol for this study was designed to meet or exceed the US EPA, OECD, and ICH Guidelines specified in the following documents:

United States Environmental Protection Agency, Title 40 Code of Federal Regulations, Part 798, Health Effects Testing Guidelines, Subpart F, Sec. 798.5265, the *Salmonella typhimurium* reverse mutation assay. Revised July 1, 2002.

OECD Guideline for the Testing of Chemicals, No. 471. Bacterial Reverse Mutation Test. Revised July 21, 1997.

International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonized Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Federal Register 61 (80):18198-18202, 1996.

The study described in this report was conducted in compliance with the following listed Good Laboratory Practice standards with the exception that the dosing solutions analysis was not conducted:

United States Environmental Protection Agency, Title 40 Code of Federal Regulations Parts 160 and 792, Revised July 1, 2002.

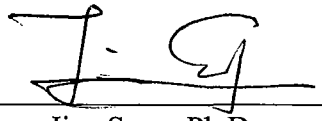
United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Revised April 1, 2003.

Japanese Ministry of Agriculture, Forestry and Fisheries, 11 NohSan, Notification No. 6283, October 1, 1999.

Japanese Ministry of Health and Welfare, Ordinance No. 21, April 1, 1997.

Japanese Ministry of International Trade and Industry, Notification No. 85, Basic Industries Bu, March 31, 1984.

Organization for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45 [ENV/MC/CHEM(98)17], Paris 1998.

Signature  10-17-08

Jian Song, Ph.D.
Study Director

Date

QUALITY ASSURANCE UNIT'S STATEMENT

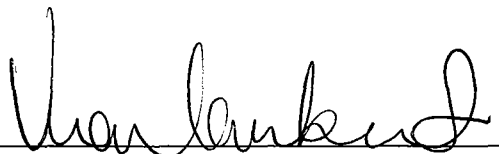
Study No.: 0985-2140
Sponsor's Test Article I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

The performance of this study was audited for adherence to the Good Laboratory Practice regulations for nonclinical laboratory studies by the Quality Assurance Unit of SITEK Research Laboratories. In this context, the facilities, equipment, personnel, methods, practices, controls, original data and reports have been inspected as per SITEK's Quality Assurance Unit's Standard Operating Procedures. The information contained within this report accurately reflects the raw data generated from this study.

Protocol Review Date: 08-26-08

The following phases were inspected for this study:

Inspection Date	Phases Inspected	Date Findings Reported to Study Director	Date Findings Reported to Management
09-03-08	Treatment of Culture	09-03-08	09-08-08
09-16-08	Workbook Audit	09-16-08	09-24-08
09-24-08	Workbook Audit	09-24-08	09-24-08
09-25-08	Draft Report Audit	09-25-08	09-26-08
10-17-08	Final Report Audit	10-17-08	10-17-08

Signature  10/17/08

Vian Lambert, B.S.
Quality Assurance Manager

Date

STUDY DIRECTOR'S SIGNATURE PAGE

This study was performed under the supervision of Jian Song, Ph.D., Study Director for *Salmonella typhimurium* and *Escherichia coli* Gene Mutation Assays, at SITEK Research Laboratories, 15235 Shady Grove Road, Suite 303, Rockville, Maryland 20850.

The Final Report for this study was written by Dr. Song and released on October 17, 2008.

Signature

Jian Song, Ph.D.
Study Director

10-17-08
Date

ABSTRACT

The test article, 3-Nitro-1,2,4-Triazol-5-one (NTO, 99.6% pure), was tested for its potential to cause mutations at the histidine operon of *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, and at the tryptophan operon of *Escherichia coli* strain WP2 uvrA. The assay was conducted using the plate incorporation method of treatment.

The test was conducted according to the method of Ames et al. in the presence and absence of metabolic activation using the S-9 fraction prepared from livers of Aroclor 1254-induced rats. The test article was tested for toxicity to strains TA100 and WP2 uvrA in a Range Finding Test at concentrations ranging from 5.0-5000 µg/plate. The test article was dissolved and subsequently diluted in Dimethyl Sulfoxide (DMSO). DMSO was used as a solvent control. The tester strains were exposed to the test article in the absence of exogenous activation and in the presence of Aroclor 1254-induced rat liver S-9 plus cofactors. Toxicity was evaluated based on: 1) reversion frequency, 2) viability, and 3) integrity of the background lawn.

The results of the Range Finding Test for TA100 indicated that the test article, NTO, was toxic at 500 µg/mL and above without activation and 5000 µg/mL with activation in regard to relative cloning efficiency. NTO was toxic to TA100 in regard to the number of revertants per plate and integrity of the background lawn at 500 µg/plate and above without activation; low revertant colonies were evident or the background lawn was absent.

The results of the Range Finding Test for WP2 uvrA indicated NTO, was toxic at 1000 µg/plate and above without activation in regard to relative cloning efficiency. NTO was toxic in regard to the number of revertants per plate and integrity of the background lawn at 5000 µg/plate without activation; low revertant colonies were evident or the background lawn was absent. With activation, the revertant were significantly decreased only at 5000 µg/plate.

The Definitive Mutation Assay, using the plate incorporation method of treatment, was performed with the four *Salmonella typhimurium* tester strains and *Escherichia coli* strain WP2 uvrA. Based on the results of the Range Finding Test, NTO was tested at concentrations of 5, 10, 50, 100 and 250 µg/plate for *Salmonella typhimurium* and 100, 250, 500, 750 and 1000 µg/plate for *Escherichia coli* without activation. With activation, the dose levels were 100, 500, 1000, 2500 and 5000 µg/plate for both *Salmonella typhimurium* and *Escherichia coli.* The results both without and with metabolic activation were negative for all strains. The background lawns were normal and the solvent and positive controls fulfilled the requirements of a valid test.

The Confirmatory Mutation Assay was also performed using the plate incorporation method of treatment. NTO was tested at concentrations of 10, 50, 100, 250 and 500 µg/plate for *Salmonella typhimurium* and 250, 500, 750, 1000 and 2500 µg/plate for *Escherichia coli* without activation. With activation, the dose levels were 100, 500, 1000, 2500 and 5000 µg/plate for both *Salmonella typhimurium* and *Escherichia coli.* The results for the Confirmatory Mutation Assay were also negative. The background lawns were normal and the solvent and positive controls fulfilled the requirements of a valid test.

The results of the Mutation Assay indicate that test article, NTO, did not induce significant increases in the frequency of revertants for all tester strains in the presence and absence of induced rat liver S-9 plus cofactors when compared to the solvent controls. Therefore, under the conditions of this test and according to the criteria set for evaluating the test results, the test article, NTO, was negative in the *Salmonella typhimurium/Escherichia coli* Plate Incorporation Mutation Assay both with and without activation.

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INTRODUCTION

This study was conducted by Jian Song, Ph.D., Shashi Sharma, B.S., Adrienne Parker, B.S., and Karen S. K. Shore, B.A., from August 28, 2008 to September 22, 2008, at SITEK Research Laboratories. The experimental procedures used to perform this study were essentially those of B. N. Ames, et al. (1), D. Maron and B. N. Ames (2), M. H. L. Green and W. J. Muriel (3), and S. Venitt and J. M. Parry (eds.) (4).

The purpose of this study was to evaluate the test article, 3-Nitro-1,2,4-Triazol-5-one (NTO), for its potential to cause mutations in the histidine operon of *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and the tryptophan operon of *Escherichia coli* strain WP2 uvrA using the Plate Incorporation method of treatment. The *Salmonella typhimurium/Escherichia coli* Plate Incorporation Mutation Assay has been used extensively and has been demonstrated to be effective in detecting mutations caused by compounds from a wide range of chemical classes (1-4). Over several years, a large database of results has been accumulated which has confirmed its ability to detect genetically active compounds of most chemical classes with high efficiency (5).

The Ames Assay, in general, is performed using either the Plate Incorporation method or Pre-Incubation method. From the regulatory point of view, both assays are equally acceptable. In the Plate Incorporation method, treatment is performed by adding either 500 µL of sterile, deionized water or 500 µL of S-9 cofactor mix to tubes containing 2.0 mL of top agar supplemented with 1X histidine-biotin or 1X tryptophan solution. Immediately thereafter, 100 µL of bacteria ($1X10^8$ bacteria) is added followed by 100 µL of the appropriate test article concentration or solvent control. Each tube is vortexed for 2-3 seconds, and the contents are evenly distributed over a Vogel-Bonner bottom agar plate. Each plate is placed on a level surface until the top agar solidifies. The plates are inverted and incubated at 37 ± 1°C for 48 to 72 hours. In the Pre-Incubation method the treatment is performed by adding either 500 µL of sterile, deionized water or 500 µL of S-9 cofactor mix to tubes followed by 100 µL of bacteria and 100 µL of the appropriate test article concentration or solvent. The tubes are incubated at 37 ± 1°C for 20-30 minutes in a shaker incubator. Finally, 2.0 mL of top agar supplemented with 1X histidine-biotin or 1X tryptophan solution is added to the tube, the contents are vortexed 2-3 seconds and spread over a Vogel-Bonner bottom agar plate. The plates are inverted and incubated at 37 ± 1°C for 48 to 72 hours. For some of the Azo and nitrosamine family of compounds, pre-incubation of the culture is required in order to be metabolized prior to plating. The Pre-Incubation method of treatment is performed at the request of the Sponsor.

The agar contains a trace of histidine that allows all the bacteria to undergo several divisions, thus producing a faint background lawn of bacteria. DNA replication is necessary in many cases for mutagenesis to occur and therefore the background lawn provides a good indicator of the inhibition of growth caused by the test chemical. Mutational events are rare, therefore it is essential that large populations of bacteria are used in mutagenicity testing. Maximum sensitivity is achieved by plating around $1X10^8$ bacteria.

The Ames Assay is the most widely used of all methods for determining the mutagenicity of chemicals. Because the bacterial strains used in this assay lack the enzymes necessary for metabolizing promutagens to ultimate mutagens, rat liver S-9 induced with Aroclor 1254 was added as a substitute for mammalian metabolism. This assay detects point mutations only and measures reverse mutation from acid auxotrophy to prototrophy. In this method, the bacterial strains used carry base substitution or frame shift mutations in operons coding for synthesis of specific amino acids. Therefore, these mutants (unlike their wild-type counterparts) cannot synthesize all their required amino acids from inorganic sources of nitrogen, being auxotrophic for the specific amino acids histidine and tryptophan. This assay determines whether the test article can reverse the effect of the pre-existing mutation by introducing a second mutation. When the cultures are exposed to a mutagen, some of the bacteria undergo genetic changes due to chemical interactions resulting in reversion of the bacteria to a non-histidine-requiring state or non-tryptophan-requiring state. The reverted bacteria will then grow in the absence of exogenous histidine or tryptophan thus providing an indication of the potential of the test chemical to cause mutation. Multiple tester strains are necessary because different strains are mutated by a different class (or different classes) of compound. The genotypes of the strains are verified concurrently.

The following are the details of possible mutations in the different strains (4):

Bacterial Strain	Mutation	Rfa	UvrB	R Factor (pKM101)	Type of Mutation
TA98*	HISD 3052	Yes	Yes	Yes	Frame shift
TA100**	HIS G46	Yes	Yes	Yes	Base Pair Substitution Frame shift
TA1535**	HIS G46 B-P	Yes	Yes	No	Base Pair Substitution
TA1537	HISC 3076	Yes	Yes	No	Frame shift
E. coli	Trp-	Yes	No (uvrA)	No	Base Pair Substitution

* TA98 was derived from TA1538 (pKM101 plasmid added).

** TA100 was derived from TA1535 (pKM101 plasmid added).

rfa - Defective lipopolysaccharide coat. More permeable to chemicals. (Sensitive to crystal violet.)

uvrB - Reduced error-free repair of some types of DNA damage. (Sensitive to UV light.)

R Factor (pKM101) - Increases sensitivity by enhancing error-prone DNA repair. (Ampicillin resistant if plasmid present.)

uvrA - Less DNA repair.

MATERIALS

TEST ARTICLE

1. Name:	3-Nitro-1,2,4-Triazol-5-one (NTO)
2. CAS No.:	932-64-9
3. Provided by:	US Army Center for Health Promotion and Preventive Medicine Aberdeen Proving Ground, MD
4. Batch/Lot No.:	BAE 07B 305-001
5. Physical Appearance:	White Powder
6. Shipping Conditions:	Room Temperature
7. Date Received:	July 23, 2008
8. Storage Conditions:	Refrigerated (1 - 5° C)
9. Purity:	99.6%
10. Expiration Date:	Not Available

CONTROL ARTICLES

Positive Controls

The positive control chemicals used for the tester strains in the presence and absence of exogenous metabolic activation are presented below:

Strain	S-9	Chemical	Concentration (µg/plate)
TA98	-	2-NF (2-Nitrofluorene)	5.0
TA98	+	2-AA (2-Aminoanthracene)	2.5
TA100	-	NaAz (Sodium Azide)	1.0
TA100	+	2-AA (2-Aminoanthracene)	2.5
TA1535	-	NaAz (Sodium Azide)	1.0
TA1535	+	2-AA (2-Aminoanthracene)	2.5
TA1537	-	9-AA (9-Aminoacridine)	75
TA1537	+	2-AA (2-Aminoanthracene)	5.0
WP2 uvrA	-	MMS (Methyl Methanesulfonate)	4000
WP2 uvrA	+	2-AA (2-Aminoanthracene)	20

The following is the information for each of the positive controls used in this assay:

Chemical	*Source	CAS No.	Lot No.	Storage Conditions	Expiration Date
2-AA	Aldrich	613-13-8	15216JA	1-5°C	08-29-09
9-AA	Aldrich	52417-22-8	1126KD	1-5°C	10-24-11
2-NF	Aldrich	607-57-8	092138A	1-5°C	03-23-12
NaAz	Sigma	26628-22-8	073K0119	1-5°C	03-23-12
MMS	Aldrich	66-27-3	06823KH	1-5°C	06-02-13

* SIGMA-ALDRICH, St. Louis, MO 63178.

The positive controls 2-AA, 9-AA, and 2-NF were dissolved in DMSO. NaAz and MMS were dissolved in sterile deionized distilled water. Multiple vials of the above mentioned positive controls were prepared and frozen at -70 °C ± 10 were used in this assay. The source, lot number and expiration date of the DMSO used to prepare the positive controls are presented below:

Source: Sigma Chemical Company
St. Louis, MO 63178

Lot No.: 10585CH

Storage Conditions: Room Temperature

Expiration Date: January 31, 2012

CAS No: 67-68-5

The source, batch numbers and expiration date of the sterile deionized, distilled water are presented below:

Source: SITEK

Batch No.: 99

Storage Conditions: Room Temperature

Expiration Dates: February 22, 2009

Solvent Control

The test article, NTO, was prepared and diluted in DMSO. Therefore, DMSO was used as the solvent control. The source, batch number and expiration date of the DMSO are provided above.

INDICATOR CELLS

Source

The *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 were originally obtained from Dr. Bruce N. Ames, University of California, Berkeley. The *Escherichia coli* strain WP2 uvrA was obtained from Ms. Judy Mayo of Pharmacia and Upjohn Co., Kalamazoo, Michigan.

CULTURE CONDITIONS

The cells were grown in Oxoid Nutrient Broth No. 2 (Oxoid LTD, Hampshire, England) in a shaker incubator rotating at approximately 120 rpm and maintained at a temperature of 37 ± 1°C. Stock cultures of the tester strains were cryopreserved at SITEK Research Laboratories. Scrapes from the cryopreserved stock were used to initiate the overnight cultures for the test.

METABOLIC ACTIVATION SYSTEM

For the activated portion of the range finding and mutation assays, the cells were exposed to the test article in conjunction with an exogenous metabolic activation system consisting of Aroclor-induced rat liver S-9 in 0.154M KCl plus cofactors (S-9 mix). The components of the standard S-9 mix were 8mM $MgCl_2$, 33mM KCl, 5mM glucose-6-phosphate, 4mM NADP, 100mM sodium phosphate buffer (pH 7.4), and 10% rat liver homogenate prepared from Aroclor 1254-induced, Sprague-Dawley rats. The S-9 batches used in this study were also evaluated for sterility, protein content and promutagen activity. Dilutions of the S-9, ranging from 0.2% to 10% in S-9 mix were tested for their ability to activate benzo(α)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to *Salmonella* strain TA100.

Source:	Molecular Toxicology, Inc., Boone, NC 28607
Inducing Agent:	Aroclor 1254
S-9 Lot No.:	2174
Protein Content:	33.4 mg/mL
Storage Conditions:	≤ -70°C
Expiration Date:	August 9, 2009

Detailed information concerning the S-9 batch used in the Assay is provided in Appendix V.

EXPERIMENTAL PROCEDURES

DOCUMENTATION

The materials, experimental procedures used in the performance of the study, experimental results and methods used in the evaluation of the results were documented in the study workbook.

TEST SYSTEM IDENTIFICATION

Plate Incorporation Method

The Plate Incorporation method is performed by adding either 500 µL of sterile deionized, water or 500 µL of S-9 cofactor mix to tubes containing 2.0 mL of top agar supplemented with 1X histidine-biotin or 1X tryptophan solution. Immediately thereafter, 100 µL of respective bacteria is added followed by 100 µL of the appropriate test article concentration or solvent. Each tube is vortexed for 2-3 seconds, and the contents are evenly distributed over a Vogel-Bonner bottom agar plate. Each plate is placed on a level surface until the top agar solidified. The plates are inverted and incubated at 37 ± 1°C for 48 to 72 hours.

Labeling Plates for the Mutation Assay

A sufficient number of Vogel-Bonner agar plates was removed from refrigerated storage and allowed to warm to room temperature. Each plate was then labeled with the following information: SITEK's test article number, experiment phase, presence or absence of rat liver S-9 mixture, concentration level code, and strain code. The following strain and concentration level codes were used:

Strain Codes:

1 = TA98 3 = TA1535 5 = WP2 uvrA
2 = TA100 4 = TA1537

Concentration Level Codes:

0 = Solvent for the Test Article
1 = 1st or highest Test Article concentration level
2 = 2nd Test Article concentration level
3 = 3rd Test Article concentration level
4 = 4th Test Article concentration level
5 = 5th Test Article concentration level or lowest Test Article concentration level for the Mutation Assays
6 = 6th Test Article concentration level
7 = 7th Test Article concentration level or lowest Test Article concentration level for the Range Finding Test.

In addition to the above, Mutation Assay viability plates that contained 10X histidine-biotin or 10X tryptophan were designated with the prefix "T".

Labeling Positive Control Plates

Vogel-Bonner agar plates were removed from refrigerated storage and allowed to warm to room temperature. Triplicate sets were labeled with the test article number, identity and concentration of the particular positive control, experimental phase, strain code, and the presence or absence of rat exogenous metabolic activation.

Labeling Tester Strain Titer Plates

Each tester strain titer plate was labeled with the following information: SITEK test article number, tester strain identity, and experimental phase and the prefix T.

Labeling Tester Strain Characterization Plates

Histidine Requirement

A single histidine-biotin plate was divided into four zones by drawing horizontal lines on the bottom of the plate with a marking pen and labeling each zone with a different *Salmonella* tester strain. A biotin-only control plate was labeled in a similar manner.

rfa Mutation

Nutrient agar plates were labeled with the *Salmonella* tester strain identification and "CV" (crystal violet).

R-Factor

A single ampicillin agar plate was labeled in a similar manner as the histidine-biotin plate.

Tryptophan Requirement

A tryptophan plate and a Vogel-Bonner agar control plate were labeled with the code for strain WP2 uvrA and used for confirmation of the tryptophan requirement.

SOLUBILITY TEST

Two small aliquots of the test article were placed in glass tubes and H_2O was added to one and DMSO was added to the other in 0.1 mL increments until dissolved.

PREPARATION OF TEST CULTURES

The methods used for the cryopreservation and cultivation of the tester strains are the procedures used by B. N. Ames et al. (1) as modified by D. Maron and B. N. Ames (2).

Inoculation Procedures

Frozen ampules of strains TA98, TA100, TA1535, TA1537 and WP2 uvrA for the Mutation Assay were removed from liquid nitrogen and placed into crushed dry ice to prevent thawing. Scrapes were made using the tip of a sterile pipette, and these scrapes were transferred to a shaker flask containing approximately 50 mL of sterile Oxoid Nutrient Broth No. 2. The strains were incubated on a shaker at approximately 120 rpm and 37 ± 1°C. The *Salmonella* strains were removed approximately 8-12 hours after the unit started and the *E. coli* strain was removed after approximately 4-6 hours.

Harvesting Overnight Cultures

Before starting the experiment, the cultures were sampled and their percent transmittance (%T) was determined using a spectrophotometer set to a wavelength of 650 nm.

When the desired cell density of approximately $5x10^8$ to $1x10^9$ cells/mL (represented by a %T of between 25% and 10%, Optical Density of 0.6-1.0) was achieved, the cultures were placed on wet ice or kept at 1-5°C until needed.

PREPARATION OF METABOLIC ACTIVATION SYSTEM

The S-9 cofactor mix was prepared as follows: For each mL of S-9 cofactor mix required, 0.335 mL of sterile deionized, distilled water was combined with 0.5 mL of 0.2M sodium phosphate buffer (pH 7.4), 0.04 mL of a 0.1M NADP solution, 5.0 µL of 1M glucose-6-phosphate, and 0.02 mL of a 0.4M $MgCl_2$/1.65M KCl salt solution. This mixture was maintained on ice until just prior to use, whereupon 0.10 mL of S-9 in 0.154M KCl was added to the mixture.

PREPARATION OF TEST ARTICLE DOSING SOLUTIONS

For the Range Finding Test and Definitive and Confirmatory Mutation Assays the test article was dissolved and diluted in the elected solvent in glass tubes. All the test article and control substance preparations and treatments were done under UV filtered lights to avoid possible problems of photoinactivation. The concentration and stability of the test article under experimental conditions was not determined.

RANGE FINDING TEST

In order to determine the toxicity of the test article and to select appropriate test article concentrations for the Definitive Mutation Assay, a Range Finding Test was performed using strains TA100 and WP2 uvrA. The two strains have been successfully used and are sufficient to approximate the range of toxicity of the test article. Seven concentrations of the test article ranging from 5.0-5000 µg/plate were evaluated with and without induced rat liver S-9, using one plate per concentration.

Spontaneous Reversion Frequency

Treatment was performed by adding either 500 µL of sterile deionized, distilled water or 500 µL of S-9 cofactor mix to tubes containing 2.0 mL of top agar supplemented with 1X histidine biotin or 1X tryptophan solution. Immediately thereafter, 100 µL of TA100 or WP2 uvrA was added followed by 100 µL of the appropriate test article concentration or solvent. Each tube was vortexed for 2-3 seconds, and the contents were evenly distributed over a Vogel-Bonner bottom agar plate. Each plate was placed on a level surface until the top agar solidified. The plates were inverted and incubated at 37 ± 1°C for 48 to 72 hours.

Viable Count Determination

Treatment and incubation were performed as described in the preceding paragraphs, except that approximately 250-500 cells of TA100 or WP2 uvrA were added to top agar supplemented with 10X histidine-biotin or 10X tryptophan solution.

After the incubation period was completed, the plates, starting with the highest test article concentration, were observed for the presence of precipitate. Plates having no interfering precipitate were counted for revertant colonies using an automatic colony counter (ARTEK Counter, Model 880, Manassas, Virginia 20110). Three counts were taken by rotating the plate on the counter stage and the median count was entered into a validated, MS Office Excel spreadsheet program designated as "2140A.xlw".

The background lawn was also evaluated. The following notations were used for the precipitate and background lawn evaluation:

Chemical Precipitate:

NP = No precipitate present.

SP = Slight precipitate - Noticeable compound on the plate; however, no influence on automated plate counting.

MP = Moderate precipitate - Moderate precipitate requiring hand counting for colony enumeration.

HP = Heavy precipitate – Large amount of compound on the plate rendering hand counting difficult.

Background Lawn Evaluation:

NL = Normal, healthy microcolony lawn.

SR = A noticeable thinning of the microcolony lawn compared to that of the solvent control plates.

MR = Marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the solvent control plates.

ER = Extreme thinning of the microcolony lawn and a large increase in the size of the microcolonies compared to the solvent control plates.

AB = Absence of any microcolony bacterial lawn.

OP = Obscured by precipitate.

Determination of Relative Cloning Efficiency

The corrected viability counts from each concentration with and without activation in *Salmonella* strain TA100 and in *Escherichia coli* strain WP2 uvrA were compared with the respective solvent control viability counts. The resulting ratio is the Relative Cloning Efficiency (RCE) and was converted into a percentage, and the data were included in the Range Finding Test results. Relative Colony Efficiency measures the toxicity of test article in terms of cell viability. Generally, diluted cultures are treated at various test article concentrations and mixed with top agar containing higher concentration of respective amino acids (10X histidine-biotin or tryptophan). All viable bacteria are able to make countable colony. It is desirable, if possible, to test one or two higher concentrations around 50% toxicity level (reduction of RCE by 50% in comparison to concurrent control) in the mutation assays. This is not valid for the non-toxic test compound. Relative Colony Efficiencies are not determined during the Definitive and Confirmatory Mutation Assays as the range of toxicity information is already available from the Range Finding Assay.

MUTATION ASSAYS

Definitive Mutation Assay

Concentrations for the Definitive Mutation Assay were selected based on the results of the Range Finding Test. The Definitive Mutation Assay was performed with the four *Salmonella typhimurium* tester strains (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* strain WP2 uvrA using the plate incorporation method of treatment. Based on the results of the Range Finding Test, the test article was tested at concentrations of 5, 10, 50, 100 and 250 µg/plate for *Salmonella typhimurium* and 100, 250, 500, 750 and 1000 µg/plate for *Escherichia coli* without activation.

With activation, the dose levels were 100, 500, 1000, 2500 and 5000 µg/plate for both *Salmonella typhimurium* and *Escherichia coli.* Treatment was performed by adding either 500 µL of sterile deionized, distilled water or 500 µL of rat S-9 cofactor mix to tubes containing 2.0 mL of top agar supplemented with 1X histidine-biotin or 1X tryptophan solution. Immediately thereafter, 100 µL of strains TA98, TA100, TA1535, TA1537 or WP2 uvrA were added, followed by 100 µL of the appropriate test article concentration or solvent. The positive controls were treated with 100 µL of the appropriate stock solutions. Each tube was vortexed for 2-3 seconds and the contents were evenly distributed over a Vogel-Bonner bottom agar plate. Each plate was placed on a level surface until the top agar solidified. The plates then were inverted and incubated at 37 ± 1°C for approximately 48 - 72 hours.

Tester Strain Titer Determination

Each tester strain was diluted to determine the approximate number of viable cells delivered to the assay plates. Therefore, approximately 250-500 cells were added to top agar supplemented with 10X histidine-biotin or 10X tryptophan solution. Each tube was vortexed for 2-3 seconds and the contents were evenly distributed on bottom agar plates. The plates were incubated at 37 ± 1°C for approximately 48 to 72 hours.

Tester Strain Characterization

All of the *Salmonella typhimurium* strains used in the assay were confirmed for the histidine requirement and the rfa mutation. In addition, strains TA98 and TA100 were tested for the presence of the pKM101 plasmid. *Escherichia coli* strain WP2 uvrA was confirmed for the tryptophan requirement.

Histidine or Tryptophan Requirement

A streak of each tester strain was made by dipping a flamed wire loop into the appropriate undiluted tester strain suspension and drawing it across the surface in the appropriate region of a labeled histidine-biotin or tryptophan plate, as well as control plates. The plates were incubated at 37 ± 1°C for approximately 12 to 24 hours.

rfa Mutation

For each of the *Salmonella* tester strains, a 100 µL aliquot of the undiluted culture was added to a tube containing 2.0 mL of 1X histidine-biotin solution in top agar. Each tube was vortexed for 2-3 seconds, and the contents were poured onto an appropriately labeled nutrient agar plate. After allowing the plate to solidify, a sterile disc was aseptically placed in the center of the agar overlay. Ten µL of a 1.0 mg/mL crystal violet solution was then added to the disc. The plates were incubated at 37 ± 1°C for approximately 12 to 24 hours.

R-Factor Plasmid

A streak of each of the *Salmonella* tester strains was made by dipping a flamed wire loop into the appropriate suspension and drawing it across the surface in the appropriate region of an ampicillin plate. The plates were incubated at 37 ± 1°C for approximately 12 to 24 hours.

uvrB Deletion

After the cryopreservation of the *Salmonella typhimurium* strains and the *E. coli* strain, the stock ampules were checked for uvrB deletion. For each of the *Salmonella* tester strains, a 100 µL aliquot of the undiluted culture was added to a tube containing 2.0 mL of 1X histidine-biotin solution top agar. One hundred µL of the *E. coli* strain was added to a tube containing 2.0 mL of 1X tryptophan solution top agar. Each tube was vortexed for 2-3 seconds, and the contents were poured onto an appropriately labeled nutrient agar plate. After allowing the plate to solidify, half of the plate was covered with foil. The plates were placed under UV light for thirty seconds and then incubated at 37 ± 1°C for approximately 12 to 24 hours.

Evaluation of Assay Results

After the incubation period was completed, the plates, starting with the highest test article concentration, were observed for the presence of precipitate. Plates were counted for the frequency of revertant colonies using an ARTEK counter, model 880. Three counts were taken by rotating the plate on the counter stage and the median count was entered into a validated, MS Office Excel spreadsheet program designated as "2140B.xlw".

The background lawn was also evaluated. The same notations as in the Range Finding Test were used to evaluate the precipitate and background lawn.

Evaluation of Tester Strain Characterization

The requirement for histidine or tryptophan was demonstrated by the growth of the tester strains on plates supplemented with histidine or tryptophan and the lack of growth on the control plates.

The presence of the rfa mutation was evaluated by measuring the zone of inhibition around the crystal violet disc. A zone ≥ 12 mm in diameter was evidence of appropriate inhibition.

The presence of the pKM101 plasmid was demonstrated by the growth of strains TA98 and TA100 and the lack of growth of strains TA1535 and TA1537 streaked on ampicillin plates.

Tabulation of Colony Counts

The colony counts provided by the automatic colony counter or by hand count were raw counts and were not corrected to reflect actual counts. Correction of the counts was performed by computer. The data tables presented in Appendix I contain the corrected values. The correction factor was determined by comparing a wide range of manual and automatic counts, as described in SITEK's SOP No. 21.0. The relationship was linear, and the counts were corrected by using the following formula:

Corrected Count = (Raw Counts) (1.0571607) + 3.09496

Confirmatory Mutation Assay

To confirm the results of the Definitive Assay, Confirmatory Mutation Assays were performed using the plate incorporation method at test article concentrations of 10, 50, 100, 250 and 500 µg/plate for *Salmonella typhimurium* and 250, 500, 750, 1000 and 2500 µg/plate for *Escherichia coli* without activation. With activation, the dose levels were 100, 500, 1000, 2500 and 5000 µg/plate for both *Salmonella typhimurium* and *Escherichia coli.*. All test article concentrations, including the controls, were tested in triplicate.

CRITERIA FOR A VALID ASSAY

The following criteria were used as guidelines in evaluating the acceptability of the Mutation Assay. Because it is impossible to formulate criteria that would apply to every configuration of data generated by the assay, the Study Director was responsible for the ultimate decision regarding the acceptability of the results.

Solvent Control Cultures

The mean reversion frequency (number of colonies on Agar plates) of the test article solvent control plates for each tester strain should fall within the following ranges:

TA98	30 ± 15	WP2 uvrA	15 ± 10
TA100	100 ± 70		
TA1535	20 ± 15		
TA1537	15 ± 12		

Positive Controls

The results for the positive control cultures were considered acceptable if the treated strains had a mean reversion frequency that was three times or higher, than the mean reversion frequency of the solvent control plates.

Tester Strain Characterization

All of the *Salmonella typhimurium* strains were confirmed positive for histidine dependence. *Escherichia coli* strain WP2 uvrA was confirmed positive for tryptophan dependence.

All of the *Salmonella typhimurium* strains were confirmed positive for the rfa mutation as evidenced by sensitivity to crystal violet.

The R-factor strains, TA98 and TA100, were confirmed positive for the pKM101 plasmid as evidenced by ampicillin resistance.

The titer of the stock cultures for each strain indicated that the stock cultures contained greater than 0.5×10^9 bacteria per mL.

EVALUATION OF TEST RESULTS

The following criteria were used as guidelines in evaluating the results of the Mutation Assay for a negative, positive or equivocal response. Because it is impossible to write criteria that would apply to every configuration of data generated by the assay, the Study Director was responsible for the ultimate decision concerning the results.

Criteria for a Negative Response

A response was considered to be negative if all of the strains treated with the test article had mean reversion frequencies that were less than twice that of the mean reversion frequencies of the corresponding solvent control plates in TA98 and TA100 and less than three times in TA1535, TA1537 and WP2 uvrA, and there was no evidence of a concentration-dependent response.

Criteria for a Positive Response

A response was considered to be positive if either strain TA98 or TA100 exhibited a mean reversion frequency that was at least double the mean reversion frequency of the corresponding solvent control in at least one concentration, or if either strain TA1535, TA1537 or WP2 uvrA exhibited a three-fold increase in the mean reversion frequency compared to the solvent control in at least one concentration. In addition, the response must have been concentration-dependent or increasing concentrations of the test article must have showed increasing mean reversion frequencies. In evaluating the results, consideration was given to the degree of toxicity exhibited by the concentration causing the 2 to 3-fold or greater increase in reversion frequency and the magnitude of the increase in reversion frequency.

Criteria for an Equivocal Response

A response was considered equivocal if it did not fulfill the criteria of either a negative or a positive response and/or the Study Director did not consider the response to be either positive or negative.

ARCHIVES

All of the raw data, documentation, protocol, protocol amendments/deviations, and final report along with an electronic file containing the data tables and final report of the study, will be maintained for 10 years in SITEK Research Laboratories' Archives at 15235 Shady Grove Road, Suite 303, Rockville, Maryland 20850.

RESULTS

SOLUBILITY TEST

3-Nitro-1,2,4-Triazol-5-one (NTO) was tested for solubility in H_2O and DMSO. The solubility in H_2O was only 14.28 mg/mL. It was soluble in DMSO at about 500 mg/mL and formed a clear yellow solution.

RANGE FINDING TEST

Summaries of the results of the Range Finding Test are presented in Tables 1 and 2 (Appendix I). The individual plate counts and background lawn evaluations are presented in Appendix II.

TA100:

The Relative Cloning Efficiencies (RCEs) at the concentrations of 5.0 to 5000 µg/plate without activation were from 2% to 87%. The revertants were significantly decreased at 500 µg/plate and above and the background lawns were absent at 5000 µg/plate. In the activation system, the RCEs at the concentrations of 5.0 to 5000 µg/plate were 37% to 109%. The significant decreased RCE (<50%) was only found at 5000 µg/plate. The revertants were not significantly decreased at any dose levels. No precipitate was observed at any of the test concentrations.

WP2 uvrA:

The Relative Cloning Efficiencies (RCEs) at the concentrations of 5.0 to 5000 µg/plate without activation were from 1% to 155%. The RCEs were 16% at 1000 µg/plate and 1% at 5000 µg/plate. The revertants were significantly decreased at 5000 µg/plate and the background lawn was also absent. In the activation system, the RCEs were from 55% to 110% at the concentrations from 5.0 to 5000 µg/plate. The revertants were significantly decreased only at 5000 µg/plate. No precipitate was observed at any of the test concentrations.

MUTATION ASSAYS

Definitive Mutation Assay

Summaries of the results of the Definitive Mutation Assay are presented in Tables 3 and 4 in Appendix I. The individual plate counts and background lawn evaluations are presented in Appendix II.

The Definitive Mutation Assay, using the plate incorporation method of treatment, was performed with the four *Salmonella* tester strains (TA98, TA100, TA1535, and TA1537) and with *E. coli* strain WP2 uvrA. Based on the results of the Range Finding Test the test article was tested at concentrations of 5, 10, 50, 100 and 250 µg/plate for *Salmonella typhimurium* and 100, 250, 500, 750 and 1000 µg/plate for *Escherichia coli* without activation. With activation, the dose

levels were 100, 500, 1000, 2500 and 5000 µg/plate for both *Salmonella typhimurium* and *Escherichia coli.*. The revertants from all of the test article-treated plates for all strains were not significantly greater than those of their corresponding solvent controls. The background lawns were normal for all concentrations. Based on these results, the Definitive Mutation Assay was negative. Both the solvent and positive controls fulfilled the requirements of the test.

Confirmatory Mutation Assay

The Confirmatory Mutation Assay was performed again using the plate incorporation method of treatment. The same strains used for the Definitive Mutation Assay were used for the Confirmatory Mutation Assay. NTO was tested at concentrations of 10, 50, 100, 250 and 500 µg/plate for *Salmonella typhimurium* and 250, 500, 750, 1000 and 2500 µg/plate for *Escherichia coli* without activation. With activation, the dose levels were 100, 500, 1000, 2500 and 5000 µg/plate for both *Salmonella typhimurium* and *Escherichia coli.*. Summaries of the results of the Confirmatory Mutation Assay are presented in Tables 5 and 6 in Appendix I. The individual plate counts and background lawn evaluations are presented in Appendix II. As in the Definitive Assay all strains treated with test article had revertant counts that were not significantly greater than those of their corresponding solvent controls. The background lawns were normal at all concentrations tested.[1] Therefore, the results were negative. The solvent and positive controls for all data presented fulfilled the requirements of the test.

SITEK's historical data for positive and solvent controls are presented in Appendix IV.

ANALYSIS OF DOSING SOLUTIONS

The Sponsor did not elect to have dosing solutions analyzed.

[1] It was necessary to repeat the Confirmatory Assay for Strains TA100 without activation, because the mean reversion frequencies of positive control were not three times or greater than the mean reversion frequencies of the test article solvent control plates.

CONCLUSIONS

The test article, 3-Nitro-1,2,4-Triazol-5-one (NTO, 99.6% pure) was tested in the *Salmonella typhimurium/Escherichia coli* Plate Incorporation Mutation Assay in the presence and absence of induced rat liver S-9. Definitive and Confirmatory Assays were performed.

The results of the Mutation Assays indicate that test article, NTO, did not induce significant increases in the revertant frequencies for the tester strains TA98, TA100, TA1535, TA1537, and WP2 uvrA in the presence and absence of induced rat liver S-9 plus cofactors when compared to the solvent controls.

Therefore, under the conditions of this study, the test article, NTO, was negative in the *Salmonella typhimurium/Escherichia coli* Plate Incorporation Mutation Assay both with and without activation.

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5. Jagannath, D.R. & Brusick, D.J. (1983) Mutagens and Carcinogens in Bacteria. In A Guide to General Toxicology (eds. Homburger, F., Hayes, J.A. & Pelikan, E.W.), S. Karger AG, P.O. Box, CH-4009 Basel, Switzerland, pp 344-364.

APPENDIX I

DATA TABLES

TABLE 1

SALMONELLA TYPHIMURIUM PLATE INCORPORATION MUTATION ASSAY
RANGE FINDING TEST RESULTS

SPONSOR: US Army CHPPM
EXPERIMENT NO.: A1
TEST ARTICLE: NTO
SITEK STUDY NO.: 0985-2140
SOLVENT: DMSO
STRAIN: TA100

WITHOUT ACTIVATION						WITH S-9 ACTIVATION					
Test Article Conc. µg/Plate	No. of Revertants/ Plate	Chem. PPT. Eval.*	Background Lawn Evaluation**	No. of Viable Colonies/ Plate	Relative Cloning Efficiency (RCE)	Test Article Conc. µg/Plate	No. of Revertants/ Plate	Chem. PPT. Eval.*	Background Lawn Evaluation**	No. of Viable Colonies/ Plate	Relative Cloning Efficiency (RCE)
5.0	137	NP	NL	265	87%	5.0	131	NP	NL	324	109%
10	114	NP	NL	237	78%	10	141	NP	NL	312	105%
50	113	NP	NL	160	52%	50	137	NP	NL	305	103%
100	97	NP	NL	175	57%	100	170	NP	NL	301	101%
500	61	NP	NL	28	9%	500	166	NP	NL	286	96%
1000	55	NP	SR	13	4%	1000	162	NP	NL	265	89%
5000	8	NP	AB	7	2%	5000	124	NP	NL	111	37%
SOLV. CONT.	113	NP	NL	305	100%	SOLV. CONT.	126	NP	NL	297	100%

$$RCE = \frac{\text{No. of Colonies in Test Plates}}{\text{No. of Colonies in Solvent Control Plates}} \times 100$$

* Chemical Precipitate Evaluation
NP = No precipitate
SP = Slight precipitate; noticeable precipitate on the plate, but no interference with automated plate counting
MP = Moderate precipitate; marked precipitate necessitating hand counting for colony enumeration
HP = Heavy precipitate; large amount of precipitate rendering hand counting difficult or impossible

** Background Lawn Evaluation
NL = Normal, healthy microcolony lawn
SR = Noticeable thinning of the microcolony lawn compared to control
MR = Marked thinning of the microcolony lawn and increase in size of microcolonies compared to control
ER = Extreme thinning of the microcolony lawn and large increase in size of microcolonies compared to control
AB = Absence of microcolonies
OP = Obscured by precipitate

Verified by: QA: VC SD: JS

TABLE 2

ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
RANGE FINDING TEST RESULTS

SPONSOR: US Army CHPPM
EXPERIMENT NO.: A1
TEST ARTICLE: NTO
SITEK STUDY NO.: 0985-2140
SOLVENT: DMSO
STRAIN: WP2 uvrA

WITHOUT ACTIVATION						WITH S-9 ACTIVATION					
Test Article Conc. µg/Plate	No. of Rever-tants/ Plate	Chem. PPT. Eval.*	Back-ground Lawn Evalu-ation**	No. of Viable Colo-nies/ Plate	Rela-tive Cloning Effi-ciency (RCE)	Test Article Conc. µg/Plate	No. of Rever-tants/ Plate	Chem. PPT. Eval.*	Back-ground Lawn Evalu-ation**	No. of Viable Colo-nies/ Plate	Rela-tive Cloning Effi-ciency (RCE)
5.0	26	NP	NL	617	155%	5.0	36	NP	NL	886	110%
10	19	NP	NL	579	145%	10	31	NP	NL	853	105%
50	31	NP	NL	596	150%	50	54	NP	NL	878	109%
100	22	NP	NL	588	148%	100	31	NP	NL	844	104%
500	23	NP	NL	479	120%	500	31	NP	NL	848	105%
1000	19	NP	NL	63	16%	1000	31	NP	NL	802	99%
5000	5	NP	AB	4	1%	5000	15	NP	NL	442	55%
SOLV. CONT.	22	NP	NL	398	100%	SOLV. CONT.	50	NP	NL	809	100%

$$RCE = \frac{\text{No. of Colonies in Test Plates}}{\text{No. of Colonies in Solvent Control Plates}} \times 100$$

* Chemical Precipitate Evaluation
NP = No precipitate
SP = Slight precipitate; noticeable precipitate on the plate, but no interference with automated plate counting
MP = Moderate precipitate; marked precipitate necessitating hand counting for colony enumeration
HP = Heavy precipitate; large amount of precipitate rendering hand counting difficult or impossible

** Background Lawn Evaluation
NL = Normal, healthy microcolony lawn
SR = Noticeable thinning of the microcolony lawn compared to control
MR = Marked thinning of the microcolony lawn and increase in size of microcolonies compared to control
ER = Extreme thinning of the microcolony lawn and large increase in size of microcolonies compared to control
AB = Absence of microcolonies
OP = Obscured by precipitate

Verified by: QA: VL SD: JS

TABLE 3
SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
DEFINITIVE MUTATION ASSAY RESULTS - WITHOUT ACTIVATION

SPONSOR: U S Army CHPPM
EXPERIMENT NO.: B-1
TEST ARTICLE: NTO
SITEK STUDY NO.: 0985-2140
SOLVENT: DMSO
CONC. IN: µg/plate

S. typhimurium		Average No. of Revertants Per Plate						
		Positive Control	Solvent Control	Concentration per plate 5	10	50	100	250
STRAIN: TA98	REVERTANTS	633	29	36	32	27	32	36
DATE PLATED:	STD. DEV.	47	7	4	5	2	4	10
09/03/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 9.100E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	306	105	115	122	116	112	116
DATE PLATED:	STD. DEV.	35	2	14	13	5	7	10
09/03/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 2.880E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	234	12	7	12	12	12	12
DATE PLATED:	STD. DEV.	6	3	1	3	2	7	2
09/03/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 2.420E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	57	13	16	15	10	14	7
DATE PLATED:	STD. DEV.	16	1	4	1	2	5	2
09/03/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 7.260E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 100	250	500	750	1000
STRAIN: WP2 uvrA	REVERTANTS	558	25	33	20	37	22	30
DATE PLATED:	STD. DEV.	7	2	8	3	2	3	9
09/03/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 2.366E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

Verified by : QA: VL SD: JS

TABLE 4
SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
DEFINITIVE MUTATION ASSAY RESULTS - WITH S-9 ACTIVATION

SPONSOR: U S Army CHPPM
EXPERIMENT NO.: B-1
TEST ARTICLE: NTO
SITEK STUDY NO.: 0985-2140
SOLVENT: DMSO
CONC. IN: µg/plate

S. typhimurium		Average No. of Revertants Per Plate						
		Positive Control	Solvent Control	Concentration per plate 100	500	1000	2500	5000
STRAIN: TA98	REVERTANTS	1237	52	58	50	47	36	31
DATE PLATED:	STD. DEV.	162	10	5	11	8	3	3
09/03/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 9.100E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	1166	135	140	149	160	168	159
DATE PLATED:	STD. DEV.	69	10	11	16	5	2	68
09/03/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 2.880E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	161	33	32	29	36	43	30
DATE PLATED:	STD. DEV.	12	4	1	1	2	7	4
09/03/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 2.420E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	402	22	30	25	28	24	17
DATE PLATED:	STD. DEV.	27	3	7	3	8	2	3
09/03/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 7.260E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 100	500	1000	2500	5000
STRAIN: WP2 uvrA	REVERTANTS	235	35	26	25	22	28	21
DATE PLATED:	STD. DEV.	54	6	5	2	8	6	3
09/03/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 2.366E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn.
NP = No precipitate.

Verified by : QA: VL SD: JS

TABLE 5
SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
CONFIRMATORY MUTATION ASSAY RESULTS - WITHOUT ACTIVATION

SPONSOR: US Army, CHPPM
EXPERIMENT NO.: B-2, B-3 (TA100)
TEST ARTICLE: NTO
SITEK STUDY NO.: 0985-2140
SOLVENT: DMSO
CONC. IN: µg/plate

S. typhimurium		Average No. of Revertants Per Plate						
		Positive Control	Solvent Control	Concentration per plate 10	50	100	250	500
STRAIN: TA98	REVERTANTS	300	42	73	50	51	47	39
DATE PLATED: 9/9/2008	STD. DEV.	27	6	6	7	4	7	6
	LAWN	NL	NL	NL	NL	NL	NL	SR
CELLS SEEDED: 1.526E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100 *	REVERTANTS	272	91	103	109	130	116	84
DATE PLATED: 9/18/2008	STD. DEV.	53	16	4	25	16	7	9
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 1.162E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	86	11	13	8	11	16	19
DATE PLATED: 9/9/2008	STD. DEV.	20	2	5	2	4	0	3
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 1.100E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	48	12	13	12	11	12	11
DATE PLATED: 9/9/2008	STD. DEV.	5	3	5	5	5	4	4
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 1.118E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 250	500	750	1000	2500
STRAIN: WP2 uvrA	REVERTANTS	580	18	24	17	27	28	28
DATE PLATED: 9/9/2008	STD. DEV.	14	3	3	5	3	7	4
	LAWN	NL	NL	NL	NL	NL	NL	SR
CELLS SEEDED: 1.772E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.
* The data for Experiment No. B2 for TA100 were not valid. The data for this strain are from a repeat Confirmatory Assay; Experiment B3

Verified by: QA: VL SD: JS

TABLE 6
SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
CONFIRMATORY MUTATION ASSAY RESULTS - WITH S-9 ACTIVATION

SPONSOR: US Army, CHPPM
EXPERIMENT NO.: B-2
TEST ARTICLE: NTO
SITEK STUDY NO.: 0985-2140
SOLVENT: DMSO
CONC. IN: µg/plate

S. typhimurium		Average No. of Revertants Per Plate						
		Positive Control	Solvent Control	Concentration per plate 100	500	1000	2500	5000
STRAIN: TA98	REVERTANTS	904	42	54	51	54	52	56
DATE PLATED:	STD. DEV.	147	4	9	1	10	9	6
9/9/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 1.526E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	813	146	135	127	110	104	120
DATE PLATED:	STD. DEV.	148	4	10	6	5	10	7
9/9/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 1.162E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	119	19	20	21	17	25	26
DATE PLATED:	STD. DEV.	6	3	7	4	3	1	8
9/9/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 1.100E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	249	13	13	13	14	16	20
DATE PLATED:	STD. DEV.	30	4	2	3	1	6	7
9/9/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 1.118E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 100	500	1000	2500	5000
STRAIN: WP2 uvrA	REVERTANTS	267	29	32	29	28	20	22
DATE PLATED:	STD. DEV.	16	4	5	6	4	4	5
9/9/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 1.772E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

Verified by: QA: VC SD: JS

APPENDIX II

DETAILED PLATE COUNTS AND

BACKGROUND LAWN EVALUATION

SALMONELLA TYPHIMURIUM PLATE INCORPORATION MUTATION ASSAY
RANGE FINDING TEST COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: A1 SITEK STUDY NO.: 0985-2140
TEST ARTICLE: NTO SOLVENT: DMSO
STRAIN: TA100

WITHOUT ACTIVATION

Test Article Conc. µg/Plate	No. of Revertants Per Plate (raw)	(corrected)	Chem. PPT. Eval.*	Background Lawn Evaluation**	No. of Viable Colonies/Plate (raw)	(corrected)	Relative Cloning Efficiency (RCE)
5.0	127	137	NP	NL	248	265	87%
10	105	114	NP	NL	221	237	78%
50	104	113	NP	NL	148	160	52%
100	89	97	NP	NL	163	175	57%
500	55	61	NP	NL	24	28	9%
1000	49	55	NP	SR	9	13	4%
5000	5	8	NP	AB	4	7	2%
SOLVENT CONTROL	104	113	NP	NL	286	305	100%

$$RCE = \frac{\text{No. of Colonies in Test Plates}}{\text{No. of Colonies in Solvent Control Plates}} \times 100$$

* Chemical Precipitate Evaluation
NP = No precipitate
SP = Slight precipitate; noticeable precipitate on the plate, but no interference with automated plate counting
MP = Moderate precipitate; marked precipitate necessitating hand counting for colony enumeration
HP = Heavy precipitate; large amount of precipitate rendering hand counting difficult or impossible

** Background Lawn Evaluation
NL = Normal, healthy microcolony lawn
SR = Noticeable thinning of the microcolony lawn compared to control
MR = Marked thinning of the microcolony lawn and increase in size of microcolonies compared to control
ER = Extreme thinning of the microcolony lawn and large increase in size of microcolonies compared to control
AB = Absence of microcolonies
OP = Obscured by precipitate

SALMONELLA TYPHIMURIUM PLATE INCORPORATION MUTATION ASSAY
RANGE FINDING TEST COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: A1 SITEK STUDY NO.: 0985-2140
TEST ARTICLE: NTO SOLVENT: DMSO
STRAIN: TA100

WITH S-9 ACTIVATION

Test Article Conc. µg/Plate	No. of Revertants Per Plate (raw)	(corrected)	Chem. PPT. Eval.*	Background Lawn Evaluation**	No. of Viable Colonies/Plate (raw)	(corrected)	Relative Cloning Efficiency (RCE)
5.0	121	131	NP	NL	304	324	109%
10	130	141	NP	NL	292	312	105%
50	127	137	NP	NL	286	305	103%
100	158	170	NP	NL	282	301	101%
500	154	166	NP	NL	268	286	96%
1000	150	162	NP	NL	248	265	89%
5000	114	124	NP	NL	102	111	37%
SOLVENT CONTROL	116	126	NP	NL	278	297	100%

$$RCE = \frac{\text{No. of Colonies in Test Plates}}{\text{No. of Colonies in Solvent Control Plates}} \times 100$$

* Chemical Precipitate Evaluation
NP = No precipitate
SP = Slight precipitate; noticeable precipitate on the plate, but no interference with automated plate counting
MP = Moderate precipitate; marked precipitate necessitating hand counting for colony enumeration
HP = Heavy precipitate; large amount of precipitate rendering hand counting difficult or impossible

** Background Lawn Evaluation
NL = Normal, healthy microcolony lawn
SR = Noticeable thinning of the microcolony lawn compared to control
MR = Marked thinning of the microcolony lawn and increase in size of microcolonies compared to control
ER = Extreme thinning of the microcolony lawn and large increase in size of microcolonies compared to control
AB = Absence of microcolonies
OP = Obscured by precipitate

ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
RANGE FINDING TEST COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: A1 SITEK STUDY NO.: 0985-2140
TEST ARTICLE: NTO SOLVENT: DMSO
STRAIN: WP2 uvrA

WITHOUT ACTIVATION

Test Article Conc. µg/Plate	No. of Revertants Per Plate (raw)	(corrected)	Chem. Background PPT. Eval.*	Lawn Evaluation**	No. of Viable Colonies/Plate (raw)	(corrected)	Relative Cloning Efficiency (RCE)
5.0	22	26	NP	NL	581	617	155%
10	15	19	NP	NL	545	579	145%
50	26	31	NP	NL	561	596	150%
100	18	22	NP	NL	553	588	148%
500	19	23	NP	NL	450	479	120%
1000	15	19	NP	NL	57	63	16%
5000	2	5	NP	AB	1	4	1%
SOLVENT CONTROL	18	22	NP	NL	374	398	100%

$$RCE = \frac{\text{No. of Colonies in Test Plates}}{\text{No. of Colonies in Solvent Control Plates}} \times 100$$

* Chemical Precipitate Evaluation
NP = No precipitate
SP = Slight precipitate; noticeable precipitate on the plate, but no interference with automated plate counting
MP = Moderate precipitate; marked precipitate necessitating hand counting for colony enumeration
HP = Heavy precipitate; large amount of precipitate rendering hand counting difficult or impossible

** Background Lawn Evaluation
NL = Normal, healthy microcolony lawn
SR = Noticeable thinning of the microcolony lawn compared to control
MR = Marked thinning of the microcolony lawn and increase in size of microcolonies compared to control
ER = Extreme thinning of the microcolony lawn and large increase in size of microcolonies compared to control
AB = Absence of microcolonies
OP = Obscured by precipitate

ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
RANGE FINDING TEST COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: A1 SITEK STUDY NO.: 0985-2140
TEST ARTICLE: NTO SOLVENT: DMSO
STRAIN: WP2 uvrA

WITH S-9 ACTIVATION

Test Article Conc. µg/Plate	No. of Revertants Per Plate (raw)	(corrected)	Chem. PPT. Eval.*	Background Lawn Evaluation**	No. of Viable Colonies/Plate (raw)	(corrected)	Relative Cloning Efficiency (RCE)
5.0	31	36	NP	NL	835	886	110%
10	26	31	NP	NL	804	853	105%
50	48	54	NP	NL	828	878	109%
100	26	31	NP	NL	795	844	104%
500	26	31	NP	NL	799	848	105%
1000	26	31	NP	NL	756	802	99%
5000	11	15	NP	NL	415	442	55%
SOLVENT CONTROL	44	50	NP	NL	762	809	100%

$$RCE = \frac{\text{No. of Colonies in Test Plates}}{\text{No. of Colonies in Solvent Control Plates}} \times 100$$

* Chemical Precipitate Evaluation
NP = No precipitate
SP = Slight precipitate; noticeable precipitate on the plate, but no interference with automated plate counting
MP = Moderate precipitate; marked precipitate necessitating hand counting for colony enumeration
HP = Heavy precipitate; large amount of precipitate rendering hand counting difficult or impossible

** Background Lawn Evaluation
NL = Normal, healthy microcolony lawn
SR = Noticeable thinning of the microcolony lawn compared to control
MR = Marked thinning of the microcolony lawn and increase in size of microcolonies compared to control
ER = Extreme thinning of the microcolony lawn and large increase in size of microcolonies compared to control
AB = Absence of microcolonies
OP = Obscured by precipitate

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
DEFINITIVE MUTATION ASSAY RAW COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: B-1
TEST ARTICLE: NTO

SITEK STUDY NO.: 0985-2140
SOLVENT: DMSO
CONC. IN: µg/plate

WITHOUT ACTIVATION

S. typhimurium		Positive Control	Solvent Control	Concentration per plate 5	10	50	100	250
STRAIN: TA98	REVERTANTS	570	32	30	32	22	26	34
	PER	648	20	35	25	25	24	38
DATE PLATED:	PLATE	570	23	28	24	22	31	21
09/03/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
9.100E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	297	95	120	123	104	109	115
	PER	313	95	101	115	104	95	110
DATE PLATED:	PLATE	250	99	95	99	112	105	96
09/03/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.880E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	214	11	3	6	9	7	7
	PER	216	7	5	10	7	3	10
DATE PLATED:	PLATE	225	8	5	8	8	15	8
09/03/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.420E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	38	10	12	10	8	5	3
	PER	48	8	16	10	6	13	3
DATE PLATED:	PLATE	68	8	8	12	6	12	7
09/03/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
7.260E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 100	250	500	750	1000
STRAIN: WP2 uvrA	REVERTANTS	518	22	25	14	31	15	35
	PER	525	22	37	15	34	20	24
DATE PLATED:	PLATE	531	19	22	19	32	20	18
09/03/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.366E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
DEFINITIVE MUTATION ASSAY RAW COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: B-1
TEST ARTICLE: NTO
SITEK STUDY NO.: 0985-2140
SOLVENT: DMSO
CONC. IN: µg/plate

WITH S-9 ACTIVATION

S. typhimurium		Positive Control	Solvent Control	Concentration per plate 100	500	1000	2500	5000
STRAIN: TA98	REVERTANTS	1150	51	53	33	46	28	24
	PER	1328	53	57	50	45	33	27
DATE PLATED:	PLATE	1022	35	47	51	33	32	29
09/03/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
9.100E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	1025	114	141	124	147	158	221
	PER	1145	128	122	154	146	155	121
DATE PLATED:	PLATE	1130	133	126	136	154	155	101
09/03/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.880E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	137	32	28	24	30	36	27
	PER	150	26	26	24	33	45	21
DATE PLATED:	PLATE	160	26	26	25	30	33	28
09/03/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.420E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	397	18	28	22	20	18	11
	PER	386	21	18	23	18	22	12
DATE PLATED:	PLATE	348	16	29	18	32	21	16
09/03/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
7.260E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 100	500	1000	2500	5000
STRAIN: WP2 uvrA	REVERTANTS	170	30	17	22	27	24	14
	PER	217	36	25	22	14	18	18
DATE PLATED:	PLATE	272	25	24	18	13	28	19
09/03/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.366E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
DEFINITIVE MUTATION ASSAY CORRECTED COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: B-1
TEST ARTICLE: NTO

SITEK STUDY NO.: 0985-2140
SOLVENT: DMSO
CONC. IN: µg/plate

WITHOUT ACTIVATION

S. typhimurium		Positive Control	Solvent Control	Concentration per plate 5	10	50	100	250
STRAIN: TA98	REVERTANTS	606	37	35	37	26	31	39
	PER	688	24	40	30	30	28	43
DATE PLATED:	PLATE	606	27	33	28	26	36	25
09/03/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
9.100E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	317	104	130	133	113	118	125
	PER	334	104	110	125	113	104	119
DATE PLATED:	PLATE	267	108	104	108	121	114	105
09/03/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.880E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	229	15	6	9	13	10	10
	PER	231	10	8	14	10	6	14
DATE PLATED:	PLATE	241	12	8	12	12	19	12
09/03/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.420E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	43	14	16	14	12	8	6
	PER	54	12	20	14	9	17	6
DATE PLATED:	PLATE	75	12	12	16	9	16	10
09/03/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
7.260E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 100	250	500	750	1000
STRAIN: WP2 uvrA	REVERTANTS	551	26	30	18	36	19	40
	PER	558	26	42	19	39	24	28
DATE PLATED:	PLATE	564	23	26	23	37	24	22
09/03/2008	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.366E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
DEFINITIVE MUTATION ASSAY CORRECTED COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: B-1
TEST ARTICLE: NTO

SITEK STUDY NO.: 0985-2140
SOLVENT: DMSO
CONC. IN: µg/plate

WITH S-9 ACTIVATION

S. typhimurium		Positive Control	Solvent Control	Concentration per plate 100	500	1000	2500	5000
STRAIN: TA98	REVERTANTS	1219	57	59	38	52	33	28
	PER	1407	59	63	56	51	38	32
DATE PLATED:	PLATE	1084	40	53	57	38	37	34
09/03/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
9.100E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	1087	124	152	134	158	170	237
	PER	1214	138	132	166	157	167	131
DATE PLATED:	PLATE	1198	144	136	147	166	167	110
09/03/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.880E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	148	37	33	28	35	41	32
	PER	162	31	31	28	38	51	25
DATE PLATED:	PLATE	172	31	31	30	35	38	33
09/03/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.420E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	423	22	33	26	24	22	15
	PER	411	25	22	27	22	26	16
DATE PLATED:	PLATE	371	20	34	22	37	25	20
09/03/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
7.260E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 100	500	1000	2500	5000
STRAIN: WP2 uvrA	REVERTANTS	183	35	21	26	32	28	18
	PER	232	41	30	26	18	22	22
DATE PLATED:	PLATE	291	30	28	22	17	33	23
09/03/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.366E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
CONFIRMATORY MUTATION ASSAY RAW COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: B-2, B-3 (TA100) SITEK STUDY NO.: 0985-2140
TEST ARTICLE: NTO SOLVENT: DMSO
CONC. IN: µg/plate

WITHOUT ACTIVATION

S. typhimurium		Positive Control	Solvent Control	Concentration per plate 10	50	100	250	500
STRAIN: TA98	REVERTANTS	309	38	60	40	49	40	39
	PER	262	42	72	52	44	48	28
DATE PLATED:	PLATE	270	31	65	41	42	36	35
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	SR
CELLS SEEDED:								
1.526E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100*	REVERTANTS	305	92	91	120	129	99	85
	PER	253	93	98	107	102	112	69
DATE PLATED:	PLATE	205	66	95	74	129	109	77
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.162E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	99	9	5	4	12	12	12
	PER	76	6	14	7	5	12	17
DATE PLATED:	PLATE	61	8	8	5	6	12	15
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.100E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	41	11	4	6	12	11	7
	PER	39	8	11	13	5	10	4
DATE PLATED:	PLATE	47	7	12	6	5	5	11
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.118E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 250	500	750	1000	2500
STRAIN: WP2 uvrA	REVERTANTS	531	12	22	9	25	21	20
	PER	551	13	21	18	20	31	23
DATE PLATED:	PLATE	555	18	17	13	24	20	27
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	SR
CELLS SEEDED:								
1.772E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.
* The data for Experiment No. B2 for TA100 were not valid. The data for this strain are from a repeat Confirmatory Assay; Experiment B3.

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
CONFIRMATORY MUTATION ASSAY RAW COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: B-2
TEST ARTICLE: NTO

SITEK STUDY NO.: 0985-2140
SOLVENT: DMSO
CONC. IN: µg/plate

WITH S-9 ACTIVATION

S. typhimurium		Positive Control	Solvent Control	Concentration per plate 100	500	1000	2500	5000
STRAIN: TA98	REVERTANTS	818	34	43	46	38	45	47
	PER	733	35	43	46	51	38	57
DATE PLATED:	PLATE	1005	41	58	44	57	55	47
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.526E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	724	133	124	112	106	106	118
	PER	653	140	116	119	101	93	107
DATE PLATED:	PLATE	923	133	134	122	96	88	108
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.162E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	105	18	14	22	14	21	23
	PER	115	12	10	14	10	21	13
DATE PLATED:	PLATE	109	15	23	15	16	20	28
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.100E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	262	6	12	9	11	9	16
	PER	205	9	8	12	9	18	9
DATE PLATED:	PLATE	230	13	8	7	9	8	23
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.118E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 100	500	1000	2500	5000
STRAIN: WP2 uvrA	REVERTANTS	263	22	31	22	27	20	22
	PER	253	24	29	31	22	12	20
DATE PLATED:	PLATE	234	29	23	20	21	15	13
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.772E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
CONFIRMATORY MUTATION ASSAY CORRECTED COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: B-2, B-3 (TA100) SITEK STUDY NO.: 0985-2140
TEST ARTICLE: NTO SOLVENT: DMSO
CONC. IN: µg/plate

WITHOUT ACTIVATION

S. typhimurium		Positive Control	Solvent Control	Concentration per plate 10	50	100	250	500
STRAIN: TA98	REVERTANTS	330	43	67	45	55	45	44
	PER	280	47	79	58	50	54	33
DATE PLATED:	PLATE	289	36	72	46	47	41	40
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	SR
CELLS SEEDED:								
1.526E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100*	REVERTANTS	326	100	99	130	139	108	93
	PER	271	101	107	116	111	121	76
DATE PLATED:	PLATE	220	73	104	81	139	118	84
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.162E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	108	13	8	7	16	16	16
	PER	83	9	18	10	8	16	21
DATE PLATED:	PLATE	68	12	12	8	9	16	19
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.100E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	46	15	7	9	16	15	10
	PER	44	12	15	17	8	14	7
DATE PLATED:	PLATE	53	10	16	9	8	8	15
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.118E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 250	500	750	1000	2500
STRAIN: WP2 uvrA	REVERTANTS	564	16	26	13	30	25	24
	PER	586	17	25	22	24	36	27
DATE PLATED:	PLATE	590	22	21	17	28	24	32
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	SR
CELLS SEEDED:								
1.772E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.
*The data for Experiment No. B2 for TA100 were not valid. The data for this strain are from a repeat Confirmatory Assay; Experiment B3.

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
CONFIRMATORY MUTATION ASSAY CORRECTED COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: B-2 SITEK STUDY NO.: 0985-2140
TEST ARTICLE: NTO SOLVENT: DMSO
CONC. IN: µg/plate

WITH S-9 ACTIVATION

S. typhimurium		Positive Control	Solvent Control	Concentration per plate 100	500	1000	2500	5000
STRAIN: TA98	REVERTANTS	868	39	49	52	43	51	53
	PER	778	40	49	52	57	43	63
DATE PLATED:	PLATE	1066	46	64	50	63	61	53
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.526E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	768	144	134	121	115	115	128
	PER	693	151	126	129	110	101	116
DATE PLATED:	PLATE	979	144	145	132	105	96	117
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.162E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	114	22	18	26	18	25	27
	PER	125	16	14	18	14	25	17
DATE PLATED:	PLATE	118	19	27	19	20	24	33
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.100E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	280	9	16	13	15	13	20
	PER	220	13	12	16	13	22	13
DATE PLATED:	PLATE	246	17	12	10	13	12	27
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.118E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 100	500	1000	2500	5000
STRAIN: WP2 uvrA	REVERTANTS	281	26	36	26	32	24	26
	PER	271	28	34	36	26	16	24
DATE PLATED:	PLATE	250	34	27	24	25	19	17
9/9/2008								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.772E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

APPENDIX III

SITEK'S HISTORICAL POSITIVE AND SOLVENT CONTROL DATA

SITEK RESEARCH LABORATORIES

HISTORICAL SOLVENT CONTROL DATA FOR SALMONELLA TYPHIMURIUM/E. COLI PLATE INCORPORATION/PREINCUBATION MUTATION ASSAY MUTANT EXPRESSED PER PLATE WITHOUT S-9 ACTIVATION

TA98	DMSO	ACET	CORN OIL	H_2O	SALINE
AVERAGE	23	26	26	24	26
STANDARD DEVIATION (±)	5	5	3	4	6
MINIMUM VALUE	14	11	21	19	17
MAXIMUM VALUE	36	32	31	34	35
N*	52	20	12	34	25

TA100	DMSO	ACET	CORN OIL	H_2O	SALINE
AVERAGE	57	99	77	64	74
STANDARD DEVIATION (±)	15	34	12	12	14
MINIMUM VALUE	38	26	51	45	50
MAXIMUM VALUE	132	174	118	99	132
N*	53	20	16	34	31

TA1535	DMSO	ACET	CORN OIL	H_2O	SALINE
AVERAGE	13	19	15	14	15
STANDARD DEVIATION (±)	3	5	4	4	4
MINIMUM VALUE	9	12	11	8	10
MAXIMUM VALUE	20	33	23	29	24
N*	56	19	14	38	28

TA1537	DMSO	ACET	CORN OIL	H_2O	SALINE
AVERAGE	9	9	10	8	9
STANDARD DEVIATION (±)	3	3	4	2	3
MINIMUM VALUE	2	6	3	5	4
MAXIMUM VALUE	20	16	23	12	15
N*	55	21	14	33	29

E.COLI	DMSO	ACET	CORN OIL	H_2O	SALINE
AVERAGE	14	15	14	16	15
STANDARD DEVIATION (±)	3	4	3	3	4
MINIMUM VALUE	8	8	10	11	10
MAXIMUM VALUE	24	25	19	22	25
N*	53	19	13	35	28

N* = NUMBER OF DATA POINTS.

SITEK RESEARCH LABORATORIES

HISTORICAL SOLVENT CONTROL DATA FOR SALMONELLA TYPHIMURIUM/E. COLI PLATE INCORPORATION/PREINCUBATION MUTATION ASSAY MUTANT EXPRESSED PER PLATE WITH S-9 ACTIVATION

TA98	DMSO	ACET	CORN OIL	H_2O	SALINE
AVERAGE	30	31	31	33	32
STANDARD DEVIATION (±)	6	4	4	5	7
MINIMUM VALUE	10	23	22	22	20
MAXIMUM VALUE	48	37	35	43	52
N*	53	19	12	36	25

TA100	DMSO	ACET	CORN OIL	H_2O	SALINE
AVERAGE	62	87	45	87	78
STANDARD DEVIATION (±)	18	30	12	14	15
MINIMUM VALUE	41	52	67	46	55
MAXIMUM VALUE	161	174	121	107	115
N*	53	19	16	37	31

TA1535	DMSO	ACET	CORN OIL	H_2O	SALINE
AVERAGE	12	16	15	14	15
STANDARD DEVIATION (±)	3	6	3	3	3
MINIMUM VALUE	8	9	11	7	9
MAXIMUM VALUE	21	33	25	21	23
N*	56	19	14	38	28

TA1537	DMSO	ACET	CORN OIL	H_2O	SALINE
AVERAGE	9	10	10	9	8
STANDARD DEVIATION (±)	3	3	4	3	2
MINIMUM VALUE	4	6	6	6	3
MAXIMUM VALUE	18	14	18	17	11
N*	57	19	22	34	29

E.COLI	DMSO	ACET	CORN OIL	H_2O	SALINE
AVERAGE	16	17	16	17	19
STANDARD DEVIATION (±)	3	4	5	3	5
MINIMUM VALUE	10	12	0	10	10
MAXIMUM VALUE	23	26	26	24	27
N*	53	17	13	35	20

N* = NUMBER OF DATA POINTS.

SITEK RESEARCH LABORATORIES

HISTORICAL POSITIVE CONTROL DATA FOR SALMONELLA TYPHIMURIUM/E. COLI
PLATE INCORPORATION MUTATION/PREINCUBATION ASSAY
MUTANT EXPRESSED PER PLATE
WITH AND WITHOUT ACTIVATION

WITHOUT ACTIVATION	TA98 (2NF)	TA100 (NaAz)	TA1535 (NaAz)	TA1537 (9AA)	E.COLI (MMS)
AVERAGE	618	419	338	135	444
STANDARD DEVIATION (±)	163	107	86	61	113
MINIMUM VALUE	180	233	57	24	122
MAXIMUM VALUE	1029	924	612	321	702
N*	107	107	109	107	108

WITH ACTIVATION	TA98 (2AA)	TA100 (2AA)	TA1535 (2AA)	TA1537 (2AA)	E.COLI (2AA)
AVERAGE	583	510	112	59	155
STANDARD DEVIATION (±)	324	261	49	28	83
MINIMUM VALUE	54	105	42	18	45
MAXIMUM VALUE	1732	1438	295	177	500
N*	311	110	109	110	108

N* = NUMBER OF DATA POINTS.

APPENDIX IV

STUDY PROTOCOL AND PROTOCOL AMENDMENT

SITEK RESEARCH LABORATORIES

EVALUATION OF A TEST ARTICLE IN THE *SALMONELLA TYPHIMURIUM/ ESCHERICHIA COLI* PLATE INCORPORATION MUTATION ASSAY IN THE PRESENCE AND ABSENCE OF INDUCED RAT LIVER S-9

This protocol is presented in two parts. Part One is designed to collect specific information pertaining to the test article and study. Part Two describes the study design in detail. **Please complete all bolded sections in Part One and sign section 8.0 to approve the protocol.**

PART ONE

1.0 SPONSOR

1.1 Name: US Army Center for Health Promotion and Preventive Medicine
Aberdeen Proving Ground, MD

1.2 Address: Aberdeen Proving Ground, MD 21010

1.3 Sponsor's Study Coordinator: Gunda Reddy, Ph.D., DABT

2.0 TESTING FACILITY

2.1 Name: SITEK Research Laboratories

2.2 Address: 15235 Shady Grove Road, Suite 303
Rockville, Maryland 20850

2.3 Study Director: Jian Song, Ph.D.

3.0 STUDY NUMBERS

* 3.1 Testing Facility's Study No.: 0985-2140

3.2 Sponsor's Study No.: Not Available

4.0 TEST ARTICLE

GLP's require that test article characterization information must be provided in the final report. This includes identification, lot number, purity, stability, source, and expiration date. As per regulatory requirements, lack of the above information will be cited as a GLP violation in the "Study Director's Compliance Statement" section of the final report.

* To be completed by the Testing Facility.

SITEK RESEARCH LABORATORIES

4.1 Identification

Name: 3-Nitro-1,2,4-Triazol-5-one (NTO)

Batch/Lot No.: BAE 07B 305-001

4.2 Description

Color: White

Physical Form: Powder

4.3 Analysis

Purity Information: 99.6%

Does the Sponsor require the use of a correction factor to account for impurity?

____ Yes ___X___ No

If yes, what is the correction factor? _____

Determination of the test article characteristics as defined by Good Laboratory Practices will be the responsibility of the Sponsor. The specific GLP references for U.S. agencies are: FDA = 21 CFR, 58.105; EPA TSCA = 40 CFR, 792.105 and EPA FIFRA = 40 CFR 160.105.

4.4 Stability

Storage Conditions (check one):

____ Room Temperature ___X___ Refrigerated (1-5°C)

____ Frozen (-10 to -20°C)

____ Other (please specify): _____

Expiration Date: Not Available

4.5 Preferred Solvent (check one):

___X___ H_2O ____ DMSO ____ Acetone ____ Ethanol

____ Other (please specify): _____

____ To be decided by the Testing Facility

SITEK RESEARCH LABORATORIES

4.6 Special Handling Instructions:

Use Standard Laboratory Safety Practices For Avoiding Exposure To Hazardous Substances.

5.0 REGULATORY AGENCY SUBMISSION

5.1 Test Design Specifications

This study protocol is designed to meet or exceed the US EPA, ICH and OECD Guidelines specified in the following documents (1, 2, 3):

United States Environmental Protection Agency, Title 40 Code of Federal Regulations, Part 798, Health Effects Testing Guidelines, Subpart F, Sec. 798.5265, the *Salmonella typhimurium* reverse mutation assay. Revised July 1, 2002.

OECD Guideline for the Testing of Chemicals, No. 471. Bacterial Reverse Mutation Test. Revised July 21, 1997.

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Federal Register 61 (80): 18198-18202, 1996.

5.2 Good Laboratory Practices

This study will be conducted in compliance with the following Good Laboratory Practice standards:

United States Environmental Protection Agency, Title 40 Code of Federal Regulations Parts 160 and 792. Revised July 1, 2002.

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58. Revised April 1, 2003.

Japanese Ministry of Agriculture, Forestry and Fisheries, 11 NohSan, Notification No. 6283, October 1, 1999.

Japanese Ministry of Health and Welfare, Ordinance No. 21, April 1, 1997.

Japanese Ministry of International Trade and Industry, Notification No. 85, Basic Industries Bureau, March 31, 1984.

Organisation for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45 [ENV/MC/CHEM(98)17], Paris 1998.

SITEK RESEARCH LABORATORIES

Will this study be submitted to a regulatory agency?

__X__ Yes ____ No

If so, which agency(ies)? Worldwide

6.0 TEST ARTICLE/DOSING SOLUTIONS CHARACTERIZATION

The U.S. requirements for analysis of dosing solutions are specified in: FDA = 21 CFR, 58.113; EPA TSCA = 40 CFR, 792.113; and EPA FIFRA = 40 CFR, 160.113.

Does the Sponsor want dosing solution analysis?

____ Yes ____ No**

If yes, please complete the rest of this section.

If requested by the Sponsor, SITEK Research Laboratories will determine the strength and stability of the dosing solutions. The method of analysis may be provided by the Sponsor, or if requested by the Sponsor, SITEK Research Laboratories will develop the method of analysis.

Alternatively, the Sponsor will be responsible for determining the strength and stability of the dosing solutions.

Dosing solution analysis will be performed by:

____ SITEK Research Laboratories ____ Sponsor***

What dosing solutions will be analyzed? _____

** Additional charges will apply. See Special Services price schedule.

*** **Please note: All work pertaining to this study that is performed outside of SITEK is the responsibility of SITEK's Study Director. Therefore, as required by the GLPs, all of the following must be forwarded to the Study Director:**

- **All subcontract and/or Sponsor Quality Assurance audit findings and comments.**
- **Any deviations and/or amendments, if applicable.**
- **An original or copy of the analysis report.**
- **Location of where the raw data from the analysis will be archived.**

If the subcontract work is not performed under the GLPs, a statement by the Sponsor informing SITEK's Study Director of such must be provided.

SITEK RESEARCH LABORATORIES

From the Range Finding Test?

_____ **Yes** _____ **No**

From the Assay?

_____ **Yes** _____ **No**

Which concentration(s)? _____

What amount of each concentration? _____

At what temperature should the dosing solutions be stored?

_____ **Room Temperature** _____ **Frozen (-10 to -20°C)**

_____ **Refrigerated (1-5°C)**

At what temperature should the dosing solutions be shipped?

_____ **Room Temperature** _____ **On Wet Ice**

_____ **On Dry Ice**

7.0 STUDY DATES

* 7.1 Proposed Experimental Start Date: August 28, 2008

Defined as the first date the test article is applied to the test system.

* 7.2 Anticipated Experimental Completion Date: September 25, 2008

Defined as the last date on which data are collected directly from the study.

* 7.3 Anticipated Draft Report Submission Date: October 10 , 2008

7.4 Final Report: The final report will be initiated sixty days after remittance of the draft report and issued no later than thirty days thereafter.

SITEK RESEARCH LABORATORIES

8.0 PROTOCOL APPROVAL

* _____ 8-26-08
Study Director — Date

_____ 8-26-08
Sponsor's Study Coordinator — Date

* _____ 8-26-08
Quality Assurance Manager — Date

* _____ 8-26-08
Safety Officer — Date

* To be completed by the Testing Facility.

SITEK RESEARCH LABORATORIES

STUDY DESIGN

PART TWO

9.0 PURPOSE

The purpose of this study is to evaluate the test article for its potential to cause mutations in the histidine operon of *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and the tryptophan operon of *Escherichia coli* strain WP2 uvrA.

10.0 JUSTIFICATION FOR SELECTION OF TEST SYSTEM

The *Salmonella typhimurium/Escherichia coli* Plate Incorporation Mutation Assay has been used extensively and has been demonstrated to be effective in detecting the mutagenic activity of chemicals from a wide range of classes.

11.0 ABBREVIATIONS

2-AA	-	2-Aminoanthracene
2-NF	-	2-Nitrofluorene
9-AA	-	9-Aminoacridine
DMSO	-	Dimethyl Sulfoxide
MMS	-	Methyl Methanesulfonate
NaAz	-	Sodium Azide
NADP	-	Nicotinamide-adenine Dinucleotide Phosphate
O.D.	-	Optical Density
%T	-	Percent Transmittance
S-9	-	Induced Rat Liver Homogenate

12.0 INDICATOR CELLS

12.1 Source

The *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 were obtained from Dr. Bruce N. Ames, University of California, Berkeley, California. The *Escherichia coli* strain WP2 uvrA was obtained from Ms. Judy Mayo of Pharmacia Corporation, Kalamazoo, Michigan.

12.2 Culture Conditions

The *Salmonella typhimurium* and *Escherichia coli* strains are routinely grown in Oxoid Nutrient Broth No. 2 in a shaker incubator rotating at approximately 120 rpm and maintaining a temperature of 37 ± 1°C.

12.3 Stock Cultures

The *Salmonella typhimurium* and *Escherichia coli* strains were propagated to obtain a sufficient number of cells for freezing a large number of stock ampules. The cells were cryopreserved in Oxoid Nutrient Broth No. 2 supplemented with 8-9% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen vapor phase. Scrapes from stock ampules are used to initiate the stock cultures for the test.

13.0 METABOLIC ACTIVATION

The standard rat liver S-9 prepared from male Sprague-Dawley rats with Aroclor-1254 or Phenobarbital and/or β-naphthoflavone will be used for the metabolic activation system

14.0 ROUTE OF ADMINISTRATION OF TEST ARTICLE

The test article will be administered in vitro directly or through a solvent compatible with the test cultures. This is the only route of administration available in this test system.

15.0 TEST SYSTEM IDENTIFICATION

All test plates will be labeled using an indelible pen with a code system which clearly identifies the experiment number, the SITEK test article number, controls, doses, and whether or not the plate was treated in conjunction with an exogenous activation system.

The test article will be designated by the unique four-digit number assigned by SITEK when the test article is received (e.g., 0074). The experiment phase will be designated by the letter A (Range Finding Test) or B (Mutation Assay) followed by a number designating the trial number. This will be followed by the letter N (No Activation) or S (With S-9) which will be followed by the dose and strain identification numbers. The doses will be identified by the numbers 1, 2, 3, ... indicating the highest to the lowest dose. The strain identification numbers will be as follows:

Salmonella typhimurium	*Escherichia coli*
1 = TA98	5 = WP2 uvrA
2 = TA100	
3 = TA1535	
4 = TA1537	

An example of a plate label follows:

0074B1-S-1-3

0074	=	SITEK Test Article Number
B1	=	First Mutation Assay
S	=	With S-9
1	=	Highest Test Article Dose
3	=	Strain TA1535

In addition to the above, the Range Finding Test and Mutation Assay viability plates that contain 10X (0.5mM) histidine biotin or 10X (0.5mM) tryptophan will be designated with the prefix "T".

16.0 CONTROL SUBSTANCES

16.1 Positive Controls

The positive control chemicals that will be used for the tester strains in the presence and absence of exogenous metabolic activation are presented below. The abbreviations are defined in Section 11.0.

	Strain	S-9	Chemical	Dose (µg/plate)
Salmonella typhimurium				
	TA98	-	2-NF	2.5-7.5
	TA98	+	2-AA	1.25-5.0
	TA100	-	NaAz	0.5-2.0
	TA100	+	2-AA	1.25-5.0
	TA1535	-	NaAz	0.5-2.0
	TA1535	+	2-AA	1.25-5.0
	TA1537	-	9-AA	25-75
	TA1537	+	2-AA	1.25-5.0
Escherichia coli				
	WP2 uvrA	-	MMS	2000-4000
	WP2 uvrA	+	2-AA	10-20

If necessary, other appropriate positive controls can be used with the approval of the Sponsor.

DMSO will be used to solubilize the positive controls, except for NaAz and MMS, which will be dissolved in deionized, distilled H_2O.

16.2 Solvent Control

The solvent used for dissolving the test article will be used as the solvent control. Deionized, distilled water, dimethyl sulfoxide (CAS #67-68-5), ethanol (CAS #64-17-5) and acetone (CAS #67-64-1) are some of the solvents which are compatible with this test system. If there is a need to use other solvents, the approval of the Sponsor will be obtained prior to their use.

17.0 DOCUMENTATION

All procedures, results, significant observations, and methods used for analysis of results will be documented in a study notebook. The study notebook will also include copies of the protocol, all protocol amendments and protocol deviations, study reports, and all relevant communications with the Sponsor.

18.0 EXPERIMENTAL PROCEDURE

18.1 Determination of Solubility/Miscibility

In order to determine the optimal vehicle for delivering the test article to the test system or to determine the maximum achievable concentration in the solvent requested by the Sponsor, a solubility/miscibility test may be performed, if necessary. The solvents of choice for this system are water, DMSO, acetone and ethanol. If the test article is not sufficiently soluble in any of these solvents, additional solvents will be screened.

For solid and viscous test articles, the solubility test will consist of weighing out 20- to 100-mg aliquots of test article and adding solvent in 0.1 mL increments, with thorough mixing between additions, until the test article is dissolved as determined by visual inspection or until 5.0 mL of solvent has been added to the vessel. The volume of solvent required for complete dissolution and any additional observations will be recorded in the study notebook. Test articles that do not dissolve in 5.0 mL of solvent will be visually inspected and recorded as either "not soluble," "partially soluble forming a homogeneous suspension," or "partially soluble not forming a homogeneous suspension."

For liquid test articles, a miscibility test will be conducted. 0.5 mL of solvent will be added to 0.5 mL aliquots of the test article. The resulting solution will be thoroughly mixed and observed for miscibility. The test article will be rated by visual inspection as either "not miscible," "partially miscible," or "completely miscible" in each of the four preferred solvents. The miscibility rating and any additional observations will be recorded in the study notebook.

Where solubility cannot be achieved, the test article will be delivered as a suspension in the desired vehicle. If sufficient solubility data is available, the solubility/miscibility test will not be performed.

18.2 Preparation of Test Cultures

The strains of *Salmonella typhimurium* and *Escherichia coli* will be prepared from cultures that were started from scrapes placed in Oxoid Nutrient Broth No. 2. The cultures will be placed on the shaker, and a timer turns on the incubator approximately 8-12 or 4-6 hours for *Salmonella typhimurium* or *Escherichia coli*, respectively, prior to sampling the cultures for growth determination. The incubator will be set at 120 rpm and 37 ± 1°C. Samples from each culture will be checked for Percent Transmittance (%T) at 650 nm.

Only cultures that have a %T of between 25% (O.D. 0.6) and 10% (O.D. 1.0) will be used.

18.3 Preparation of S-9 Metabolic Activation Mix

For the portion of the Range Finding Test or the Mutation Assay in which the cells are exposed to the test article in conjunction with an exogenous metabolic activation system, induced rat liver S-9 plus cofactors (S-9 mix) will be used as the activation system. The components of the standard S-9 mix will be 8mM $MgCl_2$, 33mM KCl, 5mM glucose-6-phosphate, 4mM NADP, 100mM sodium phosphate buffer (pH 7.4), and 10% rat liver S-9.

18.4 Preparation of Test Article

The desired amount of the test article as specified in the dilution scheme will be weighed or measured just prior to use in either the Range Finding Test or the Mutation Assay. The dosing solutions will be prepared by adding the appropriate volume of solvent to the test article and thoroughly mixing the resulting solution until the test article goes completely into solution or a homogeneous suspension is achieved. The remaining doses specified in the dilution scheme will be prepared by either performing a serial dilution or by varying the volume delivered from the stock concentration to the cultures. In all treatments the amount of solvent delivered to the target cultures will be limited to a level which has no cytotoxic effect on the cells. If necessary, the test article may be added directly to the top agar.

18.5 Range Finding Test

In order to determine the test article concentrations that will produce from 0-100% toxicity, a Range Finding Test will be performed with and without S-9 activation using tester strains TA100 and WP2 uvrA only. The test article will be weighed or measured, and a serial dilution will be prepared. If there are no solubility/miscibility limitations, prior knowledge of cytotoxicity indicates differently, or the Sponsor specifies differently, the treatment concentrations for solid and viscous test articles will be 5000, 1000, 500, 100, 50, 10 and 5.0 µg/plate. If the results based on the dosing regimen indicate that the threshold level of complete toxicity is below 5.0 µg/plate an additional Range Finding Test will be performed.

18.5.1 Treatment

2.0 mL aliquots of molten top agar, to which trace amounts of histidine and biotin have been added, will be dispensed to a series of culture tubes maintained at 45 ± 1°C. Treatment will be performed by adding 0.5 mL of S-9 mix or 0.5 mL of sterile, distilled, deionized water, 0.1 mL of tester strain TA100 or WP2 uvrA, and 0.1 mL of test article to the top agar. Appropriate solvent controls will also be prepared.

In addition, plates for determining viability will be prepared by plating the test article doses with a $2.0x10^5$ dilution of tester strain TA100 or WP2 uvrA in top agar containing 10X histidine-biotin or 10X tryptophan, respectively.

The contents will be mixed by vortexing the tube, and then the contents will be poured onto a bottom agar plate and evenly distributed by gently tilting and rotating the plate. The plate will be placed on a flat, level surface until solidified. After all treatment is performed, the plates will be inverted and incubated at 37 ± 1°C for 48-72 hours.

18.5.2 Determination of Toxicity

After 48-72 hours of incubation, the plates will be removed from the incubator and evaluated or placed in cold storage (1-5°C) until evaluated.

Evaluation of test article toxicity on the tester strain will be based on three end points:

1. Viability of cells plated on minimal medium plates supplemented with excess histidine-biotin or tryptophan. Toxicity will be measured as a decrease in the number of colonies per plate with increasing test article concentration.

2. The number of revertant colonies on minimal medium plates supplemented with trace amounts of histidine-biotin or tryptophan. Toxicity will be measured as a reduction in the number of revertant colonies per plate with increasing test article concentration.

3. The integrity of the background microcolony lawn. Toxicity will be measured as a thinning or disappearance of the background lawn usually occurring with an increase in the size of the remaining microcolonies relative to the control plates.

The number of revertants per plate and the number of viable colonies per plate will be determined by counting them with an automatic colony counter or by hand as described in Sections 18.6.5.1 and 18.6.5.2.

The counts will be entered directly in the Excel 2003 computer program 2140A. and the calculations will be performed. The computer printouts will be included in the study notebook.

18.6 Mutation Assay

The maximum concentration of nontoxic test articles that is tested will be 5 mg per plate, unless the Sponsor requests otherwise or precipitation of the test article on the plate warrants the use of a lower concentration. Test articles that produce a toxic effect will be tested at a maximum dose that significantly reduces the number of revertants per plate and/or causes thinning of the background lawn. Four lower doses will be selected that should not produce toxicity. Test articles that are insoluble at concentrations of 5 mg per plate or lower will be tested at a maximum dose that produces precipitate. A concentration that produces precipitate in the test system will be considered to be beyond the limits of solubility. The actual dose levels for the assay, once determined, will be added to the protocol in the form of an amendment. Each test article dose, the positive controls and solvent controls will be plated in triplicate.

18.6.1 Test Culture Preparation and Exposure

Cultures of *Salmonella typhimurium*, TA98, TA100, TA1535, TA1537, and *Escherichia coli* WP2 uvrA for use in the Mutation Assay will be prepared as described in Section 18.2. The test article will be weighed or measured, and a serial dilution will be performed as previously described in Section 18.4. 2 mL aliquots of molten top agar to which histidine and biotin or tryptophan have been added will be dispensed to a series of culture tubes maintained at 45 ± 1°C. Treatment will be performed by adding 0.5 mL of S-9 mix or 0.5 mL of sterile, distilled, deionized water, 0.1 mL of tester strain, and 0.1 mL of test article to the top agar. Appropriate solvent and positive controls will also be prepared. The contents will be mixed by vortexing the tube, and then the contents will be poured onto a bottom agar plate and evenly distributed by gently tilting and rotating the plate. The plate will be placed on a flat, level surface until solidified. After all treatment will be performed, the plates will be inverted and incubated at 37 ± 1°C for 48-72 hours.

18.6.2 Confirmation of Tester Strain Genotypes

On the same day as the plating of the Mutation Assay, the genotypes of the tester strains will be confirmed. All of the *Salmonella typhimurium* strains will be tested for histidine dependence and the rfa mutation. Each *Salmonella typhimurium* strain will be tested for the uvrB deletion after cryopreservation of the stock ampules. The tester strains TA98 and TA100 will also be tested for the pKM101 plasmid. The *Escherichia coli* WP2 uvrA strain will be tested for tryptophan dependence.

18.6.3 Tester Strain Viability Determination

After the Mutation Assay has been plated, a dilution of each tester strain will be prepared, and approximately 250-500 bacteria will be plated in top agar supplemented with 10X histidine-biotin or 10X tryptophan. These plates will be incubated for 48-72 hours, and then the total number of colonies that develop will be determined.

18.6.4 Background Lawn Evaluation

The integrity of the background microcolony lawn will be evaluated by viewing each plate with the aid of a 2X to 4X microscope. The lawns will be rated as normal, slightly reduced, markedly reduced, extremely reduced or absent.

18.6.5 Enumeration of Colonies

After 48-72 hours of incubation, the plates treated with the highest test article concentration will be observed for the presence of precipitate. If precipitate is absent, the entire assay will be counted using an automatic colony counter. If observation of the high dose plates reveals precipitate that interferes with accurate automatic counting, those plates will be counted by hand. The procedure will be repeated for each subsequent dose level or until no precipitate is evident.

18.6.5.1 Automatic Colony Counting

Each plate will be placed on the stage, and three counts are made with the automatic counter. The plate will be rotated on the stage approximately 120° between each count, and the median count will be recorded.

18.6.5.2 Hand Counting

Hand counting of colonies will be performed by marking a dot over each colony on the bottom of the plate while clicking off the counts on a digitometer. The hand count will be recorded for each plate.

The counts will be entered directly in the Excel 2003 computer program 2140B. The computer printouts will be included in the study notebook.

18.7 Confirmatory Mutation Assay

If the first Mutation Assay gives negative or equivocal results, a confirmatory Mutation Assay will be performed. The test article treatment concentrations may be altered based on the results obtained in the first Mutation Assay. On the other hand, if the results of the first Mutation Assay are clearly positive, a confirmatory Mutation Assay may or may not be performed depending on the Sponsor's instructions.

18.8 Criteria For a Valid Assay

The following criteria will be used as guidelines in determining the acceptability of the results. Since it is impossible to formulate criteria that would apply to every configuration of data generated by the Mutation Assay, the Study Director will be responsible for the ultimate decision regarding the acceptability of the results.

18.8.1 Solvent Control Cultures

The mean reversion frequency of the test article solvent control plates for each strain must fall within the range presented below.

Salmonella typhimurium Escherichia coli

TA98	30 ± 15	WP2 uvrA	15 ± 10
TA100	100 ± 70		
TA1535	20 ± 15		
TA1537	15 ± 12		

18.8.2 Positive Controls

The results for the positive control cultures will be considered acceptable if the treated strains have mean reversion frequencies that are three times or greater than the mean reversion frequencies of the test article solvent control plates.

18.8.3 Tester Strain Characterization

1. All of the *Salmonella typhimurium* strains will be confirmed positive for histidine dependence and the *Escherichia coli* strain for tryptophan dependence.

2. All of the *Salmonella typhimurium* strains will be confirmed positive for the rfa mutation as evidenced by sensitivity to crystal violet.

3. The R-factor strains, TA98 and TA100, will be confirmed positive for the pKM101 plasmid as evidenced by ampicillin resistance.

4. The titer of the stock cultures of each strain will indicate that the stock cultures contained greater than $0.5x10^9$ cells/mL.

18.9 Evaluation of Test Results

The following criteria will be used as guidelines in evaluating the results of the Mutation Assay for a negative, positive or equivocal response. Since it is impossible to write criteria that would apply to every configuration of data generated by the Mutation Assay, the Study Director will be responsible for the ultimate decision in the evaluation of the results. The factors considered in making the decision will be discussed in the report.

18.9.1 Criteria for a Negative Response

A response will be considered negative if 1) strains TA98 and TA100 have mean reversion frequencies that are less than twice that of the mean reversion frequencies of the corresponding solvent control plates, 2) strains TA1535, TA1537 and WP2 uvrA have mean reversion frequencies less than three times that of the corresponding solvent control plates, and 3) there is no evidence of a dose-dependent response.

18.9.2 Criteria for a Positive Response

A response will be considered positive if either strain TA98 or TA100 has a dose that produces a mean reversion frequency that is greater than or equal to two times the mean reversion frequency of the corresponding solvent control plates or if either strain TA1535, TA1537 or WP2 uvrA has a dose producing a three-fold or greater increase in the mean reversion frequency compared to the solvent control frequency. In addition, the response must be dose-dependent or increasing concentrations of the test article must show increasing mean reversion frequencies. In evaluating the results, consideration will be given to the degree of toxicity exhibited by the dose causing the two-fold/three-fold or greater increase in reversion frequency and the magnitude of the increase in reversion frequency.

18.9.3 Criteria for an Equivocal Response

A response will be considered equivocal if it does not fulfill the criteria of either a negative or a positive response and/or the Study Director does not consider the response to be either positive or negative.

In addition, if either strain TA1535, TA1537 or WP2 uvrA has a dose producing a twofold increase in mean reversion frequency compared to the solvent control frequency and there is a dose-dependent response at lower concentrations in this strain, then this result will be considered equivocal and the test may be repeated after consultation with the Sponsor.

19.0 PROTOCOL AMENDMENTS AND DEVIATIONS

If changes in the approved protocol are necessary, such changes will be documented in the form of protocol amendments and protocol deviations. Protocol amendments will be generated when changes in the protocol are made prior to performing a study or part of a study affected by the changes. In such cases, a verbal agreement to make such changes will be made between the Study Director and the Sponsor. These changes and the reasons for them will be documented and attached to the protocol as an addendum. Protocol deviations will be generated when the procedures used to perform the study do not conform to the approved protocol. The Sponsor will be informed of these deviations, and as soon as practical, such changes along with their reasons or explanations will be documented and kept in the study notebook.

20.0 REPORT OF RESULTS

20.1 Content

The results of the study will be submitted to the Sponsor in the form of a final report. A draft report will be submitted before the final report is issued. The final report will be initiated sixty days after remittance of the draft report and issued no later than thirty days thereafter. The report will include, but not be limited to, the following:

1. Name and address of the testing facility and the dates on which the study was initiated and completed, terminated or discontinued.

2. Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

3. Methods used to analyze the data.

4. The test and control substances.

5. Description of the methods used to perform the study.

6. The data, mean plate counts, +/- SD, and any observations regarding toxicity and precipitate.

7. The name and signature of the Study Director and the names of other technical personnel who participated in performing the study.

8. The location where the raw data and reports are to be stored.

9. A statement from the Quality Assurance Unit.

20.2 Changes and Corrections to the Final Report

All changes to the final report will be in the form of a report amendment which will include the reason(s) for the change, and the amendment will be added to the final report as an addendum.

21.0 ARCHIVES

The raw data, electronic file containing the data tables, documentation, protocol and final report of the study will be maintained in the SITEK Research Laboratories Archives, 15235 Shady Grove Road, Suite 303, Rockville, Maryland, according to the terms and conditions of the study.

22.0 REFERENCES

1. Ames, B. N., J. McCann and E. Yamasaki. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mut. Res., 31:347-367, 1975.

2. Maron, D., and B. N. Ames. Revised methods for the *Salmonella* mutagenicity test. Mut. Res., 113:173-215, 1983.

3. Green, M. H. L., and W. J. Muriel. Mutagen testing using trp+ reversion in *Escherichia coli*. In: B. J. Kilbey, et al. (eds.), Handbook of Mutagenicity Test Procedures, pp. 65-94, Elsevier North Holland Biomedical Press, Amsterdam, 1977.

4. Venitt, S., and J. M. Parry (eds.). Mutagenicity testing: A practical approach. IRL Press, Oxford, England and Washington, D.C., 1984.

PROTOCOL AMENDMENT 1

Amendment No.:	1
Sponsor:	US Army Center for Health Promotion and Preventive Medicine Aberdeen Proving Ground, MD 21010
Testing Facility:	SITEK Research Laboratories 15235 Shady Grove Road, Suite 303 Rockville, Maryland 20850
SITEK's Study No.:	0985-2140
Sponsor's Study No.:	N/A
Test Article I.D.:	3-Nitro-1,2,4-Triazol-5-one (NTO)
Protocol Title:	Evaluation of a Test Article in the *Salmonella Typhimurium/Escherichia Coli* Plate Incorporation Mutation Assay in the Presence and Absence of Induced Rat Liver S-9
Amendment No. 1:	Protocol page 2, section 4.5: Preferred solvent was changed from H_2O to DMSO.
Reason for Amendment No. 1:	The test article was not sufficiently soluble in H_2O.

APPROVED:

Jian Song, Ph.D.
Study Director

8.28.08
Date

PROTOCOL AMENDMENT 2

Amendment No.:	2
Sponsor:	US Army Center for Health Promotion and Preventive Medicine Aberdeen Proving Ground, MD 21010
Testing Facility:	SITEK Research Laboratories 15235 Shady Grove Road, Suite 303 Rockville, Maryland 20850
SITEK's Study No.:	0985-2140
Sponsor's Study No.:	N/A
Test Article I.D.:	3-Nitro-1,2,4-Triazol-5-one (NTO)
Protocol Title:	Evaluation of a Test Article in the *Salmonella Typhimurium/Escherichia Coli* Plate Incorporation Mutation Assay in the Presence and Absence of Induced Rat Liver S-9

Amendment No. 2:

Protocol page 12, section 18.6: Based on the results of Range Finding Test the actual dose levels for the definitive mutation assay will be 5, 10, 50, 100 and 250 µg/plate for *Salmonella Typhimurium* and 100, 250, 500, 750 and 1000 µg/plate for *Escherichia Coli* without activation. With activation, the actual dose levels will be 100, 500, 1000, 2500 and 5000 µg/plate for both *Salmonella Typhimurium* and *Escherichia Coli.*

For the confirmatory mutation assay the actual dose levels will be 10, 50, 100, 250 and 500 µg/plate for *Salmonella Typhimurium* and 250, 500, 750, 1000 and 2500 µg/plate for *Escherichia Coli* without activation. With activation, the actual dose levels will be 100, 500, 1000, 2500 and 5000 µg/plate for both *Salmonella Typhimurium* and *Escherichia Coli.*

Reason for Amendment No. 2: Protocol page 12, section 18.6: The actual dose levels for the assay, once determined, will be added to the protocol in the form of an amendment.

APPROVED:

Jian Song, Ph.D.
Study Director

9-15-08
Date

APPENDIX V

S-9 BATCH INFORMATION

MOLTOX POST MITOCHONDRIAL SUPERNATANT (S-9) QUALITY CONTROL & PRODUCTION CERTIFICATE

LOT NO.: 2174 **SPECIES:** Rat **PREPARATION DATE:** August 9, 2007
PART NO.: 11-101 **STRAIN:** Sprague Dawley **EXPIRATION DATE:** August 9, 2009
VOLUME: 2ml **SEX:** Male **BUFFER:** 0.154 M KCl
TISSUE: Liver **INDUCING AGENT(s):** Aroclor 1254 (Monsanto KL615), 500 mg/kg i.p.
REFERENCE: Maron, D & Ames, B, *Mutat Res* **113**:173, 1983
STORAGE: At or below -70°C

BIOCHEMISTRY:

- PROTEIN

33.4 mg/ml — Assayed according to the method of Lowry et al., *JBC* **193**:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold-Induction
EROD	IA1, IA2	119.9
PROD	2B1	30.5
BROD	2B1	25.4
MROD	1A2	120.8

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* **34**:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/mg protein) were 21.3, 16.3, 51.0, & 7.3 for EROD, PROD, BROD and MROD, respectively.

BIOASSAY:

- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Triplicate plates were read after 40 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants	
TA98	TA1535
104.8	1204

The ability of the sample to activate ethidium (EtBr) EtBr/CPA/and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* **129**:299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 - 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted using duplicate plates as described by Maron & Ames, (*Mutat Res* **113**:173, 1983).

µl S9 per plate/number *his*$^+$ revertants per plate

Promutagen	0	1	5	10	20	50
BP (5 µg)	106	160	312	408	552	1410
2-AA (2.5 µg)	103	309	1248	2051	2553	2382

MOLECULAR TOXICOLOGY, INC.
157 Industrial Park Dr.
Boone, NC 28607
(828) 264-9099

Rev'd: 3.18.08

APPENDIX VI

CERTIFICATE of ANALYSIS

BAE SYSTEMS

Ordnance Systems Kingsport, Tennessee

Certificate of Analysis for: NTO Per ORDNANCE SYSTEMS SPECIFICATION

Certificate No.: BAE 1686
Customer PO No.: 2007003323 Customer Part No.: N/A

	Batch Number:	10NTO7-3
	Containers Shipped:	14
	Lot Number:	BAE07B305-001
Characteristics	**Specs**	
% Purity by HPLC	99.0 - 100.0	99.6
Acidity, % as Nitric	0.01 Max	0.00
Exotherm Onset, Deg. C	250 Min	265
% Moisture	0.05 Max	0.03
Impact,cm		52
Appearance, Crystalline Solid	White to Pale Yellow	Pale Yellow Crystalline
Workmanship	PASS	PASS

Total Drums: 14 **Total Pounds:** 800

Prepared by: Neal Roberts

Digitally signed by Neal Roberts
DN: cn=Neal Roberts, c=US, o=BAE Systems, ou=Quality Assurance, email=neal.j.roberts@baesystems.com
Date: 2008.08.04 16:48:08 -04'00'

Neal Roberts, Quality Manager
Wednesday, February 28, 2007

[redacted]

Destination Control:
These items are covered by the United States Munitions List (USML) and is therefore subject to control by the U.S. Department of State. Export requires an export license, or other approval, issued by the U.S. Department of State.